Bacillus megaterium:

Versatile Tools for Production, Secretion and Purification of Recombinant Proteins

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"Man sieht nur mit dem Herzen gut. Das Wesentliche ist für die Augen unsichtbar.
Die Zeit, die Du für Deine Rose verloren hast, sie macht Deine Rose so wichtig.
Die Menschen haben diese Wahrheit vergessen, aber Du darfst sie nicht vergessen.
Du bist zeitlebens für das verantwortlich, was Du Dir vertraut gemacht hast.
Du bist für deine Rose verantwortlich..."

„Der kleine Prinz“, Antoine de Saint-Exupery, 1943

Allen,
die an mich geglaubt haben

TABLE OF CONTENTS

ABBREVIATIONS

2D	two dimensional
A_λ	absorption at wavelength λ in nm
aa	amino acid
Amp	ampicillin
AP	alkaline phosphatase
APB	alkaline phosphatase buffer
APS	ammonium peroxodisulfate
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
BCIP	5-Brom-4-chlor-3-indolyl phosphate
CAI	codon adaptation index
CDW	cell dry weight
CHAPS	3-[(3-cholamidopropyl)-dimethyalmino]
CIP	calf intestinal alkaline phosphatase
Cml	chloramphenicol
CV	column volume
Da	dalton
DNA	deoxyribonucleic acid
D.N.S.	dinitrosalicylic acid
(d)dNTP	(di)deoxyribonucleotide triphosphate
dsDNA	double stranded DNA
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTT	1,4-dithio-D,L-threitol
EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (for example)
et al.	*et alteri* (and others)
FACS	fluorescent activated cell sorting
Fig.	figure
for	forward
FSC	frontal scatter

g	→ *centrifugation*: earth gravity
	→ *weight*: gram
GFP	green fluorescent protein
h	hour
HCDC	high cell density cultivation
HPLC	high pressure liquid chromatography
H_2O_{deion}	deionised water
IEF	isoelectric focussing
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilo base pair
K_D	dissociation constant
kDa	kilo Dalton
λ	wave length
LB	Luria Bertani
m	milli
M	molar [mol l^{-1}]
μ	micro
μ_{set}	growth rate
MALDI	matrix assisted laser desorption/ionisation
Mb	mega base pairs
MCS	multiple cloning site
min	minute
MOPS	3-(N-morpholino)-propan sulfonacid
MOPSO	3-(N-morpholino)-2-hydroxy propan sulfonacid
M_r	relative molecular mass
MS	mass spectrometry
n	nano
NBT	nitroblue tetrazolium
NIPAB	6-Nitro-3-phenylacetamido-benzoic acid
NTA	nitrilotriacetic acid
OD_λ	optical density at wavelength λ in nm
ORF	open reading frame
ori	origin of replication

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG 6000	polyethylen glycol with M_r of 6000
PI	propidium iodide
PVDF	polyvinylidene difluoride
RBS	ribosome binding side
rev	reverse
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	secretion
SP	signal peptide
SRP	signal recognition particle
SSC	side scatter
ssDNA	single stranded DNA
Tab.	table
TAE	Tris-acetate/EDTA
TAT	twin arginine translocation
TB	terrific broth
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	tetramethylen diamine
Tet	tetracycline
TEV	tobacco etch virus
T_M	hybridisation temperature
TOF	time of flight
TRIS	tris-(hydroxymethyl)-aminomethane
Triton-X100	t-octylphenoxy polyethoxy ethanol
U	unit

UV	ultraviolet
vs.	versus
v/v	volume per volume
w/v	weight per volume

I INTRODUCTION

I.1 FROM GENE TO PRODUCT

The project outlined in the following thesis was part of the Collaborative Research Centre SFB 578 (Sonderforschungsbereich 578) "Development of Biotechnological Processes by Integrating Genetic and Engineering Methods – From Gene to Product". It is based on the collaboration of different institutes from the Technical University of Braunschweig, the Helmholtz Centre for Infection Research (formerly called "Gesellschaft für Biotechnologische Forschung"), and the University of Applied Sciences of Magdeburg. One major aim of this collaboration is the investigation of a complete biotechnological production process starting with the first cloning step to the final downstream processing of the target product by using the prokaryotic host organism *Bacillus megaterium*. For this purpose, the collaboration was divided into five main project areas (Hempel, 2006):

[A] Molecular Biology of Product Formation
[B] Systems Biotechnology of Product Formation
[C] Process Technology
[D] Application Technology
[Z] Genome Project: *Bacillus megaterium*

Each of the main project areas consists of two to five sub-projects. This thesis was incorporated into the sub-project A1 "Production of recombined glycosyltransferases in *Aspergillus niger* and *Bacillus megaterium*". Aim of our sub-project was the establishment and investigation of genetic tools for *B. megaterium*. This includes vector construction for recombinant protein production, the production of the recombinant proteins in shaking flask scale as well as the purification of recombinant proteins for further applications within the Collaborative Research Centre SFB578.

I.2 *BACILLUS MEGATERIUM*

The Gram positive bacterium *Bacillus megaterium* was first described by de Bary more than one century ago (De Bary, 1884). With its eponymous size „megat(h)erium" (Greek: "big animal") of 1.5 x 4 µm, this microorganism belongs to the larger bacteria. Due to the

dimension of the vegetative form and spores, *B. megaterium* is well suited for morphological research, for example on cell-wall and cytoplasmic membrane biosynthesis, on sporulation, spore structure and organisation (Vary, 1992). In the 1960th, prior to *Bacillus subtilis* *B. megaterium* was "the" model organism for intensive studies on sporulation.

Primarily a soil bacterium, *B. megaterium* is also found in diverse environments from rice paddies to dried food, seawater, sediments, fish, normal flora, and even in bee honey (Vary, 1994). Taxonomically, *B. megaterium* is placed into the *B. subtilis* group of *Bacilli* (Priest, 1993; Vary, 1994) although there is only a small degree of relation in the genome structure between *B. megaterium* and *B. subtilis*.

Due to its application for the production of several biotechnological relevant substances, *B. megaterium* is of general interest for industry. It is completely apathogenic. In contrast to Gram negative organisms like *Escherichia coli*, it does not produce endotoxins associated with the outer membrane. These features make *B. megaterium* well applicable in food and even in pharmaceutical industry. The utilisation of a wide variety of carbon sources let this organism grow on low cost substances. *B. megaterium* does not possess any obvious alkaline proteases degrading recombinant gene products. Therefore, a large amount of intact functional proteins with only little or no degradation products are yielded (Kim, 2003; von Tersch and Robbins, 1990). This presents a further reason which favours *B. megaterium* strains for the production of recombinant proteins. A further advantage is its ability to secret proteins directly into the growth medium (Priest, 1977). To name only a few products with high industrial importance, *B. megaterium* is used for the production of several alpha- and beta-amylases which are used for starch modification in the baking industry. Furthermore, *B. megaterium* is utilised for the production of penicillin acylases which are essential for the synthesis of novel β-lactam antibiotics (Panbangred *et al.*, 2000). Neutral proteases, which are employed in the leather tanning industry in Indonesia, are also produced and secreted by *B. megaterium* (personal communication, F. Meinhardt; Münster). Moreover, *B. megaterium* is known for its ability to synthesise vitamin B_{12} aerobically and anaerobically (Raux *et al.*, 1998).

I.2.1 *Bacillus megaterium* Plasmids

Several *B. megaterium* strains are known to carry significant parts of their genetic material on plasmids (Vary, 1994). A well characterised *B. megaterium* strain harbouring several plasmids is QM B1551 (ATCC12872). Its seven indigenous plasmids show different copy numbers and comprise approximately 11 % of the total cellular DNA. The size spectrum of

those plasmids reaches from only 5.4 kb to over 165 kb (Kieselburg *et al.*, 1984). The two smallest ones replicate by the rolling circle mechanism whereas the other five are theta replication plasmids with cross-hybridisation replicons (Stevenson *et al.*, 1998). The rolling circle replicons show similarities to sequences of plasmids known from *Bacillus thuringiensis* and *Bacillus anthracis* (Scholle *et al.*, 2003). The theta replicons appear to be unique. They may form a new class of compatible replicons (Kunnimalaiyaan and Vary, 2005). Beside these different DNA sequences, the plasmids of *B. megaterium* QM B1551 carry several interesting genes, *e.g.* genes coding for proteins involved in cell division, in germination, in heavy metal resistance, in cell wall hydrolysis, or in rifampin resistance. Even a complete rRNA operon is located on one of the plasmids (Kunnimalaiyaan and Vary, 2005; Scholle *et al.*, 2003). *B. megaterium* strain PV361, a derivative of strain QM B1551 (Sussman *et al.*, 1988), lacks all seven plasmids. Surprisingly, this plasmidless strain is able to grow on rich medium and shows no differences in sporulation compared to QM B1551. Therefore, the plasmids of QM B1551 may play a role in its adaptation to various environmental conditions.
For industrial applications and research, plasmidless strains are used as hosts for efficient plasmid encoded production of foreign proteins. In comparison to *B. subtilis*, *B. megaterium* is known for its ability to stably replicate and maintain recombinant plasmids (Rygus and Hillen, 1991; Vary, 1994). Moreover, an efficient protoplast transformation system does exist (Barg *et al.*, 2005; von Tersch and Robbins, 1990).

I.2.2 *Bacillus megaterium* Producing Recombinant Proteins Using a Xylose-Inducible Promoter System

Rygus and Hillen identified a xylose-inducible promoter P_{xylA} with the according repressor protein XylR in the genome of *B. megaterium* strain DSM319 (Rygus *et al.*, 1991). This promoter is located upstream of an operon coding for the xylose isomerase XylA, the xylulokinase XylB and the xylose permease XylT. XylA and XylB are necessary for the biochemical phosphorylation of xylose to xylose-5-phosphate while XylT is responsible for the active transport of xylose into the cell. The gene encoding the repressor protein XylR is located divergently oriented upstream of this operon (**Fig. 1**) while the promoter regions of *xylR* and of the *xyl*-operon are overlapping. The regulation of the *xyl*-operon expression occurs on transcriptional level. In the absence of xylose, XylR binds to the two tandem overlapping operator sequences located in P_{xylA} and prevents transcription of the *xyl*-operon (Dahl *et al.*, 1994; Gärtner *et al.*, 1988). In the presence of xylose, the sugar binds to the repressor XylR. This results in a conformational change of XylR and its release from the

operators. In this case, the RNA-polymerase is able to recognise the promoter and initiates gene expression. An additional level of regulation is mediated by glucose (Gärtner *et al.*, 1988). A so called catabolite response element (*cre*) sequence is situated in the *xylA* open reading frame from base 23 to 200 (Rygus and Hillen, 1992). This *cis*-active *cre* element and the *trans*-active catabolite controlled protein (CcpA) are essential for catabolite regulation in *B. megaterium*. In the presence of glucose HPr, a phospho-carrier protein of the phosphoenolpyruvate:glycose phosphotransferase system (PTS), is phosphorylated at Ser-46 which enhanced CcpA-binding to the *cre* sequence (Deutscher *et al.*, 1995). Xylose induced gene expression is repressed 14-fold. In the presence of xylose and glucose as sole energy sources, *B. megaterium* shows diauxic growth. As long as glucose is consumed, expression of the *xyl*-operon is inhibited by CcpA binding to the *cre* sequence. When glucose becomes exhausted, the organism is able to switch from glucose to xylose consumption and enters a second log phase (Rygus and Hillen, 1992). Beside glucose, also other carbon-sources like fructose and mannitol are known to induce catabolite regulation.

Based on this xylose-inducible promoter, Rygus and Hillen developed a xylose-dependent plasmid-borne system for the overproduction of recombinant proteins. This plasmid encoded system includes the coding sequences for XylR, the promoter P_{xylA} and the first 195 bp of the *xylA* (*xylA'*) followed by a multiple cloning site (MCS). The designed plasmid called pWH1520 (**Fig. 1B**) was successfully used for the recombinant intracellular production of prokaryotic and eukaryotic proteins including *E. coli* β-galactosidase, *B. megaterium* glucose dehydrogenase, *Acinetobacter calcoaceticus* mutarostase, human urokinase-like plasminogen activator (Rygus and Hillen, 1991) and *Clostridium difficile* toxin A (Burger *et al.*, 2003). Toxin A, with a high molecular mass of 308,000, was produced even in a higher amount compared to recombinant production in *E. coli*. This was probably due to a higher similarity in codon usage of *toxA* to the host *B. megaterium*.

In further studies in our laboratory, the promoter region of *xylA* in pWH1520 was modified and optimised for recombinant gene expression. The *cre* sequence was eliminated and an enhanced MCS was inserted (Malten, 2002; Malten *et al.*, 2005a). The resulting plasmid pMM1520 (**Fig. 1C**) allows simple cloning of target genes by the use of 15 different DNA restriction enzyme cleavage sites located in the new designed MCS. Moreover, glucose and other sugars present in nutrient rich medium do not inhibit recombinant gene expression by catabolite control anymore. To further optimise this system, a derivative of the plasmidless *B. megaterium* strain DSM319, WH323, was constructed. This strain has a deletion in the

xylose isomerase gene *xylA* and is therefore unable to consume the inducer xylose as C-source (Rygus and Hillen, 1992).

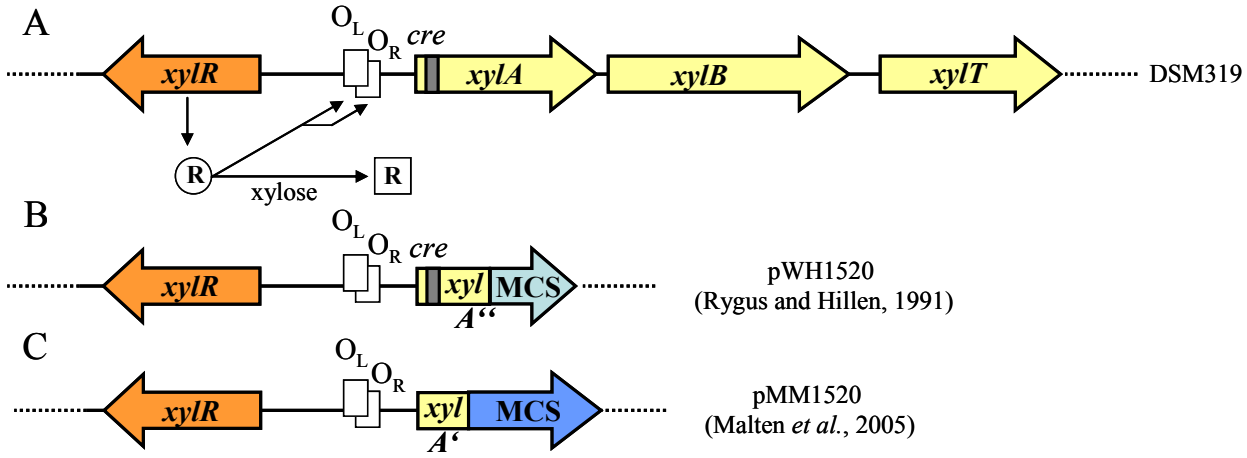


Figure 1: Genetic organisation of the *xyl*-operon from *B. megaterium* and the control elements of the *B. megaterium* expression vectors pWH1520 and pMM1520. The genes involved in the xylose utilisation in *B. megaterium* are indicated by yellow arrows. The *xylR* gene (orange) encodes the xylose repressor protein. *xylA, xylB* and *xylT* (yellow), which are co-transcribed from the xylose-inducible promoter P_{xylA}, encode the xylose isomerase, the xylulokinase and the xylose permease. The *xyl* operator region is denoted by O_LO_R (white boxes) indicating the two overlapping binding sites for XylR (Dahl *et al.*, 1994). The *cre*-element (dark grey box) indicates the catabolite response element. **(A)** Genetic organisation of the *xyl*-operon in *B. megaterium* strain DSM319. Under gene repressive conditions in the absence of xylose, XylR is in an operator binding formation (circle). In the presence of xylose the inducer interacts with XylR. This results in a conformation (squares) of XylR unable to bind to the operator (Dahl *et al.*, 1995; Schmiedel *et al.*, 1997). **(B)** Genetic organisation of the *xyl*-operon elements encoded on pWH1520 (Rygus and Hillen, 1991). The first 195 bp of *xylA*, denoted as *xylA''*, are fused to a short DNA sequence encoding the multiple cloning site (MCS, light blue). The operator sequences O_LO_R and the *cre*-box in *xylA''* are still present. **(C)** Genetic organisation of the *xyl*-operon elements encoded on pMM1520 (Malten *et al.*, 2005a). The first 15 bp of *xylA*, denoted as *xylA'*, are fused to a short DNA sequence encoding an enhanced MCS (blue). The operator sequences O_LO_R are still present while the *cre*-box was eliminated.

I.3 PROTEIN SECRETION IN *BACILLI*

Bacilli have to directed their proteins to five different (sub)cellular locations: 1. cytoplasm, 2. cytoplasmic membrane, 3. membrane/cell wall interface, 4. cell wall, and 5. environment (summarised in **Fig. 2**). The final destination is defined by protein additions and modification. These include a signal peptide, which is an amino acid sequence located at the N-terminus of the polypeptide chain, and retention signals such as transmembrane segments, lipid

modifications or cell wall binding repeats. The secretion of various proteins into the environment enables the cell to attack and utilise highly polymeric nutrients like polysaccharides, nucleic acids, peptides and lipids. After degradation into monomeric or dimeric building blocks, these are taken up. For this purpose, so called exoenzymes have to pass the hydrophobic cell membrane and the cell wall to be released into the environment. Five different secretion pathways are known in *Bacilli sp.* characterised by different kinds of signal peptides. Signal peptides differ in lengths, charge, hydrophobicity, and signal peptidase recognition sites, respectively (Tjalsma *et al.*, 2000).

The best characterised secretion pathway is the SEC- (secretion) dependent protein transport. It has been studied intensively in *E. coli* where the SEC-pathway is applied to direct proteins into the periplasm (Makrides, 1996). Studies on *B. subtilis* demonstrated that SEC-dependent secretion is similar in Gram negative and Gram positive bacteria (Tjalsma *et al.*, 2000; Tjalsma *et al.*, 2004; van Wely *et al.*, 2001). After the protein is synthesised at the ribosomes of *B. subtilis*, the N-terminal signal peptide is recognised by proteins of the SEC-system. The newly synthesised protein is kept in an unfolded state and transported through a channel in the cytoplasmic membrane into the space between membrane and cell wall. A special type I signal peptidase anchored at the surface of the cell membrane removes the signal peptide by cleavage at its recognition sequence. Chaperones in the space between cytoplasmic membrane and cell wall assist in protein folding. Finally, the protein defuses through the cell wall into the growth medium (summarised in Tjalsma *et al.*, 2004). In this thesis, the SEC-pathway was used for the secretion of heterologous proteins by *B. megaterium* and is described in more detail later on.

A further secretion pathway is the TAT-dependent protein transport which was first described for chloroplasts (Mould and Robinson, 1991). It was named the twin arginine transport (TAT) since two arginine residues are typically found in the N-terminal signal peptide of the pre-protein. Via this pathway, completely folded proteins often containing cofactors are transported into the environment (Palmer and Berks, 2003; Sanders *et al.*, 2001). A third protein transport pathway used for membrane located lipoproteins also utilises the SEC-machinery in order to pass the membrane. In such a case, the signal peptide is cleaved by a type II signal peptidase. A lipid is attached to a conserved cysteine residue, anchoring the protein in the membrane. Although some lipoprotein signal peptides contain a twin arginine motive (Jongbloed *et al.*, 2002), a transport via the TAT-pathway has not been reported so far. Fourth, pseudopilins have their own transport pathway, called Com-system. Pseudopilins are involved in competence development of *B. subtilis*. Cleavage of the signal peptide proceeds at

the cytoplasmic site of the membrane. Only four proteins (ComGC, ComGD, ComGE, ComGG) with pseudopilin signal peptides have been identified in *B. subtilis* so far (Tjalsma *et al.*, 2000). ABC-transporters form the fifth class of export pathways. Via this export mechanism, polypeptides consisting of non proteinogenic amino acids like bacteriocines or pheromones are transported into the environment.

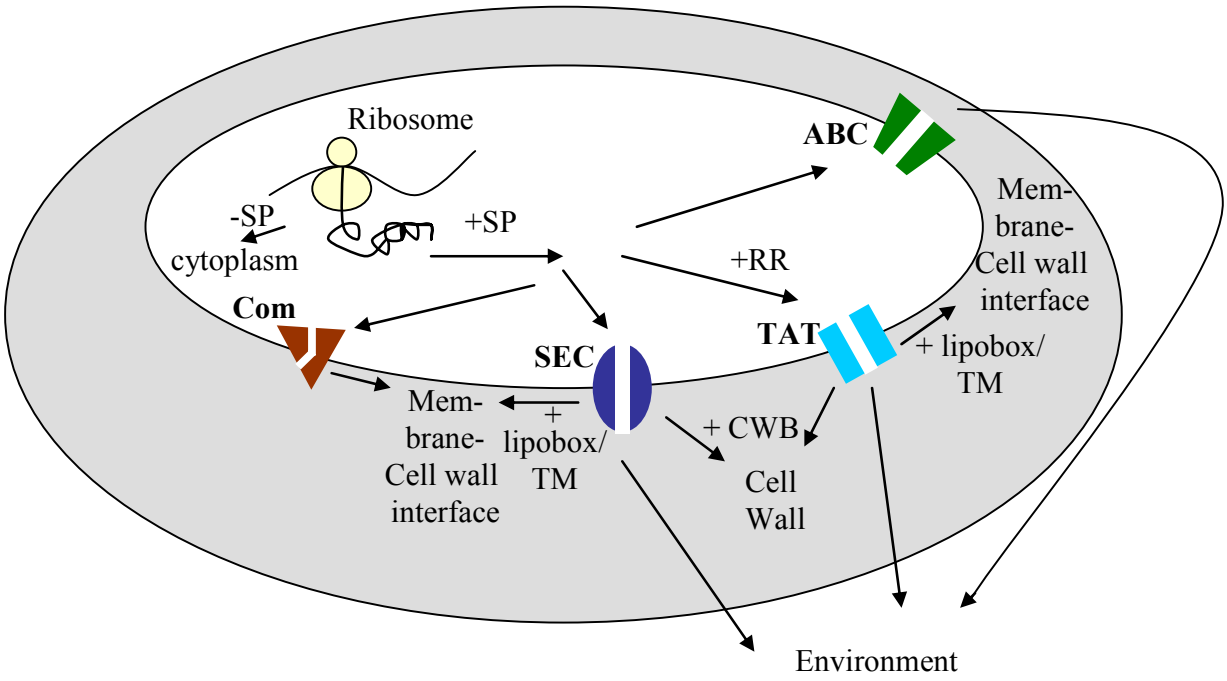


Figure 2: Protein transport pathways in *B. subtilis* (modified after Tjalsma *et al.*, 2000 and 2004). After proteins are synthesised at the ribosomes, they are sorted to various cellular destination depending on the absence (-SP) or presence (+SP) of an N-terminal signal peptide (SP) and specific retention signals. Without SP, proteins remain in the cytoplasm. With SP, proteins stay at the extracytoplasmic side of the membrane if containing a transmembrane segment (TM) or a lipid modification (lipobox). They are transported by the SEC- or the TAT-pathway. Pseudopilins are transported via the Com-system. Proteins remaining in the cell wall are exported either via the SEC- or the TAT-pathway. To be retained in the cell wall, the mature part of the protein contains so called cell wall binding repeats (CWB). Proteins can be secreted into the environment via the SEC- or the TAT-pathway or by ABC-transporters.

I.3.1 SEC-Dependent Pathway for Protein Export in *Bacilli*

In *Bacilli*, 90 % of all secreted proteins are transported via the SEC-dependent pathway demonstrating the major importance of this mechanism for the secretion of proteins (**Fig. 3**). During the formation of an exoprotein at the ribosomes, components of the SEC-system recognise the N-terminal secretion signal of the precursor form of the exoprotein. In *B. subtilis*, proteins with signal peptides of 24 to 65 amino acid in length were found

(Antelmann *et al.*, 2001). These signal peptides consist of three typical regions – an N-terminal positively charged (N-) region with up to 11 basic amino acids like arginine or lysine, followed by a hydrophobic core (H-) region containing a helix-breaking residue mostly a glycine or a proline residue, and a more hydrophilic C-terminal (C-) region. The C-region ends with the signal peptidase cleavage site. In order to pass the membrane, the protein has to remain in an unfolded conformation. Usually chaperones in the cytoplasm bind to the synthesised unfolded exoprotein in order to prevent its folding. The N-terminal signal peptide is bound by a protein-RNA-complex immediately after its translation. This signal recognition particle (SRP) consists of small cytoplasmic RNA, the GTPase Ffh and a histone-like protein HBsu. Pre-proteins bound to the SRP are targeted to the secretion apparatus with the aid of the FtsY protein. Moreover, a functional SecB analogue CsaA was found in *B. subtilis* (Mueller *et al.*, 2000). In *E. coli*, SecB is an export-dedicated cytosolic chaperone that is required for the efficient export of a subset of pre-proteins. The chaperone CsaA shows no structural homology to SecB, but has functional similarities in its binding to the unfolded signal peptide of a few pre-proteins. These pre-proteins are then transferred by CsaA to the secretion apparatus in the cytoplasmic membrane by binding to SecA. The exact mechanism is still unknown.

The pre-protein is secreted through a channel in the cytoplasmic membrane to the space between membrane and cell wall. The channel consists of the transmembrane proteins SecY, SecE and SecG. When SecE is bound to SecY, the channel is closed. If, however, the signal peptide binds to SecY and thus displaces SecE, the channel is opened for the export of the protein. SecA, an essential part of the secretion apparatus, is the so called motor protein of the secretion machine. It is only loosely associated to the SecYEG channel and found in relatively high concentrations in the cytoplasm. It receives the pre-protein from chaperones CsaA or SRP/FtsY-complex by binding to the signal peptide and guides it to the membrane. The binding of a pre-protein stimulates ATPase activity of SecA. ATP binding by SecA leads to the insertion of the C-terminus of SecA through the pore of the SecYEG complex in the membrane, resulting in the translocation of a short stretch of the pre-protein. The insertion of the C-terminus is followed by ATP hydrolysis. This leads to the release of the pre-protein and to the deinsertion of SecA. By this ATP driven cycle, the target protein is transported through the cytoplasmic membrane.

Outside the cytoplasm, a type I signal peptidase which is anchored to the cell membrane removes the signal peptide by cleavage at its recognition sequence. In *B. subtilis*, five membrane associated type I signal peptidases are known. Only two of them were reported to

be essential for the viability of *B. subtilis* (Tjalsma *et al.*, 1998). Nahrstedt *et al.* were able to identify and functionally test a type I signal peptidase of *B. megaterium*, called SipM, which is essential for the viability of *B. megaterium* (Nahrstedt *et al.*, 2004).

In secretome studies of *B. subtilis* (Antelmann *et al.*, 2001), the frequency of specific amino acid residues at the signal peptidase cleavage site of secreted proteins was studied. It revealed to be highly conserved. At the -3 position an alanine (68 %) or a valine (20 %) is found while an alanine is conserved at -1 position. Almost all amino acid residues (except cysteine and proline) are allowed at position -2 because it sticks out of the active site of the signal peptidase. Nevertheless, a preference for a serine (22 %) seems to exist. The amino acid chain of the mature protein often starts with an alanine (27 %), but besides proline and cysteine, all other amino acids are allowed (Tjalsma *et al.*, 2000).

After cleavage of the signal peptide, the unfolded protein is released into the space between cytoplasmic membrane and cell wall. As long as it stays in an unfolded conformation, it is prone to proteolytic degradation by cell wall associated proteases like WprA. In *B. subtilis*, the extracellular chaperone PrsA, a lipoprotein located on the surface of the cell membrane, assists the proteins to fold after crossing the membrane (Vitikainen *et al.*, 2001). Overproduction of PrsA in *B. subtilis* enhanced the secretion of an α-amylase of *Bacillus stearothermophilus* 4-fold (Vitikainen *et al.*, 2005). PrsA deficient mutants of *B. subtilis* show high degradation of secreted proteins (Kontinen and Sarvas, 1993).

The folded proteins diffuse through the cell wall to finally reach the surrounding culture broth. The thick cell wall of Gram positive bacteria represents a further barrier. Crosslinked peptidoglycans which function like a molecular sieve retard the passage of large proteins. Further acidic polysaccharides, the teichoic acids, deposited in this peptidoglycan layer confer a negative charge to the cell wall. These structures most likely interact with basic proteins. However, the negatively charged cell wall stores calcium ions accelerating the folding of some exported proteins (van Wely *et al.*, 2001).

After entering the surrounding culture medium, the exported protein may be attacked by extracellular proteases. *B. subtilis* secretes several alkaline serine proteases (Bolhuis *et al.*, 1999), whereas only one major extracellular protease NprM has been found and identified in *B. megaterium* so far (Wittchen and Meinhardt, 1995). Hence, secreted proteins are more stable in the medium of *B. megaterium* cultures and are open to further purification and application.

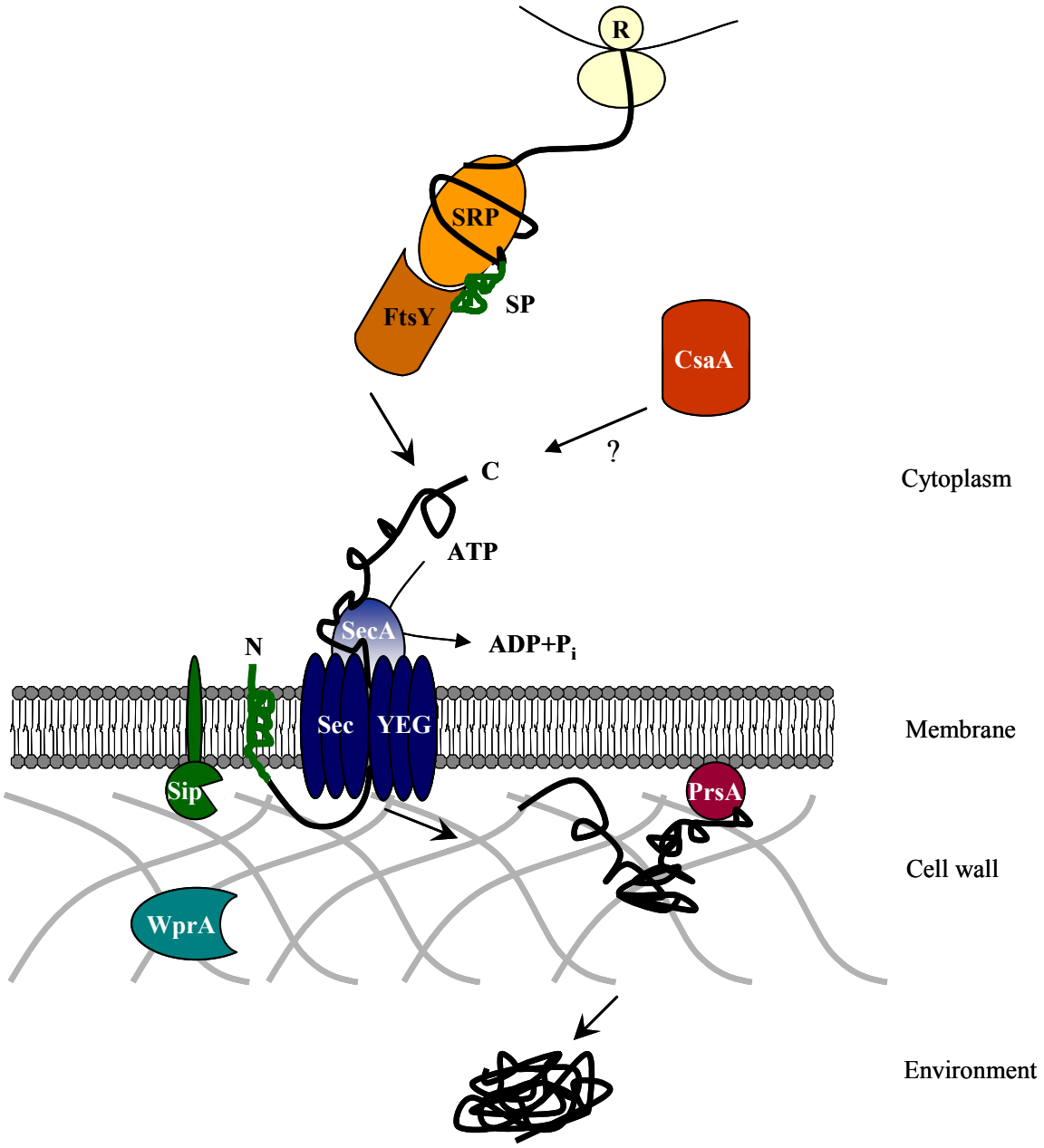


Figure 3: Components involved in the SEC-dependent protein transport in *B. subtilis* (simplified after Tjalsma *et al.*, 2004). Secretory proteins are synthesised as a precursor molecule with an N-terminal signal peptide (SP) at the ribosome (R). Cytosolic chaperones like the SRP/FtsY-complex or CsaA bind to the pre-protein to keep it in an unfolded conformation. They direct the unfolded protein to the secretion translocase in the membrane, consisting of the motor protein SecA and the three membrane proteins SecY, SecE and SecG. During or after translocation, the SP of the pre-protein is cleaved by a type I signal peptidases. The folding of several secreted proteins depends on the activity of PrsA or on other extracellular chaperones (not shown). Extracellular proteases of *B. subtilis* like WprA are involved in quality control of exported proteins. After folding, the mature protein passages through the cell wall and is released into the environment.

I.3.2 Recombinant Protein Secretion in *Bacillus megaterium*

In *B. megaterium* several naturally secreted proteins were characterised but only little research was done on the secretion of heterologous proteins. However, it gained interest as production system for polysaccharide degrading enzymes like glucanases due to its natural glucanase deficiency. One early work describes the expression of an *endo-β-1,4-glucanase* from *B. subtilis* in *B. megaterium* under control of its own promoter (Lee and Pack, 1987). In 2003, a similar report was published on the secretion of a *Bacillus circulans* endo-β-1,3-1,4-glucanase in *B. megaterium* and *B. subtilis*. The production in *B. subtilis* yielded more recombinant protein. However, as demonstrated before, the plasmid stability was higher in *B. megaterium* (Kim, 2003). Four years ago in our laboratory, first studies were started using the xylose-inducible plasmid-borne gene expression system for the production and secretion of recombinant proteins by *B. megaterium* (Malten, 2002). The production and secretion of the high molecular mass dextransucrase DsrS (M_r = 188,000) from *Leuconostoc mesenteroides* were investigated and systematically optimised (Malten, 2005; Malten *et al.*, 2005a; Malten *et al.*, 2005b). After successful production and secretion of DsrS into the growth medium of *B. megaterium* DSM319, the use of a protease deficient *B. megaterium* strain MS941 (Δ*nprM*) (Wittchen and Meinhardt, 1995) enhanced the secretion and extracellular stability of this enzyme. High cell density cultivations of *B. megaterium* resulted in up to 28,600 U l^{-1} of recombinant DsrS. Overproduction of the *B. megaterium* signal peptidase SipM further increased recombinant DsrS production and secretion. To minimise observed aggregation problems, coexpression of the *B. subtilis* extracellular chaperone *prsA* was investigated but showed no positive effect on DsrS secretion (Malten, 2005).

I.4 Recombinant Protein Production and Purification

For several decades, much effort was devoted to optimise vector systems for recombinant gene expression and protein production in various organisms. Several of these vector systems are commercially available like the pET-series for *E. coli* (Novagen; pET-System-Manuel; 11th edition) or the pMUTIN-series for *B. subtilis* (Kaltwasser *et al.*, 2002). Kaltwasser *et al.* described the construction of six *B. subtilis* vectors allowing the fusion of a target gene to six different epitopes and localisation tags. Beside these epitope tags like the Myc-epitope (Evan *et al.*, 1985) and localisation tags like fluorescence marker (Lewis and Marston, 1999), fusion to further affinity tags for recombinant protein purification is common (Terpe, 2003).

I.4.1 Protein Purification Using Affinity Tags

The production of recombinant proteins in an apparently pure form for characterisation, crystallisation or applications in the pharmaceutical industry has become a major task in research. Using *E. coli* as expression host, fast and simple purification of recombinant proteins using so called affinity tags can be achieved. Established vector systems are commercially available for *E. coli* (Makrides, 1996). Appropriate expression vectors provide the genetic setup for the in frame fusion of peptides or proteins with high ligand binding specificity to the target protein. During the last years, several epitope peptides and proteins have been developed for over-production and purification of recombinant proteins. These affinity ligands are proven to have a minimal effect on the tertiary structure and the biological activity of the fusion protein. In addition to large fusion peptides like the glutathione S-transferase (GST) or the maltose-binding protein (MBP), small peptide tags like polyhistidine (His-tag) or a streptavidin binding polypeptide (Strep-tag) are commonly used (Lichty *et al.*, 2005).

Histidine is the amino acid that exhibits the strongest interaction with immobilised metal ion matrices. Electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilised transition metal ion (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) (Porath *et al.*, 1975). This leads to a strong interaction between the metal ion and the amino acid side chain. The method of protein purification by histidine residues was first described in 1987 (Hochuli *et al.*, 1987). His_6-tagged proteins bind to the Ni^{2+} nitrilotriacetic acid (Ni-NTA) under native condition in low- or high-salt buffers. After binding, the target protein can be eluted by an increasing imidazole gradient or by decreasing the pH to 5.0.

The StrepII-tag, a derivative of the Strep-tag, is an eight amino acid peptide with the sequence "Trp-Ser-His-Pro-Gln-Phe-Glu-Lys". It was developed for the purification of fusion proteins on Strep-Tactin columns. Strep-Tactin is a variant of the protein streptavidin and exhibits a 10-fold higher affinity to the StrepII-tag than streptavidin does (Schmidt and Skerra, 1993; Voss and Skerra, 1997). StrepII-tagged proteins are bound under physiological buffer conditions in the biotin binding pocket of the Strep-Tactin and can be eluted with biotin derivatives like desthiobiotin.

Once the fusion protein has been isolated, the presence of affinity tags may be unrequested for further applications. The most popular method to remove the tag involves the use of a specific protease like the Factor Xa (Jenny *et al.*, 2003) or the tobacco etch virus (TEV) protease (Kapust *et al.*, 2002). This involves the insertion of an unique amino acid sequence specific for each protease between affinity tag and target protein (Terpe, 2003). Pure target protein

may be obtained by using an appropriate protease which carries the same affinity tag as the desired protein does. By applying a second affinity chromatographic step, the cleaved affinity tag and the tagged protease bind to the immobilised binding partner while the target protein is found in the flow-through.

A scheme of a typical purification amplification used for His_6- or StrepII-tagged proteins is given in **Fig. 4**.

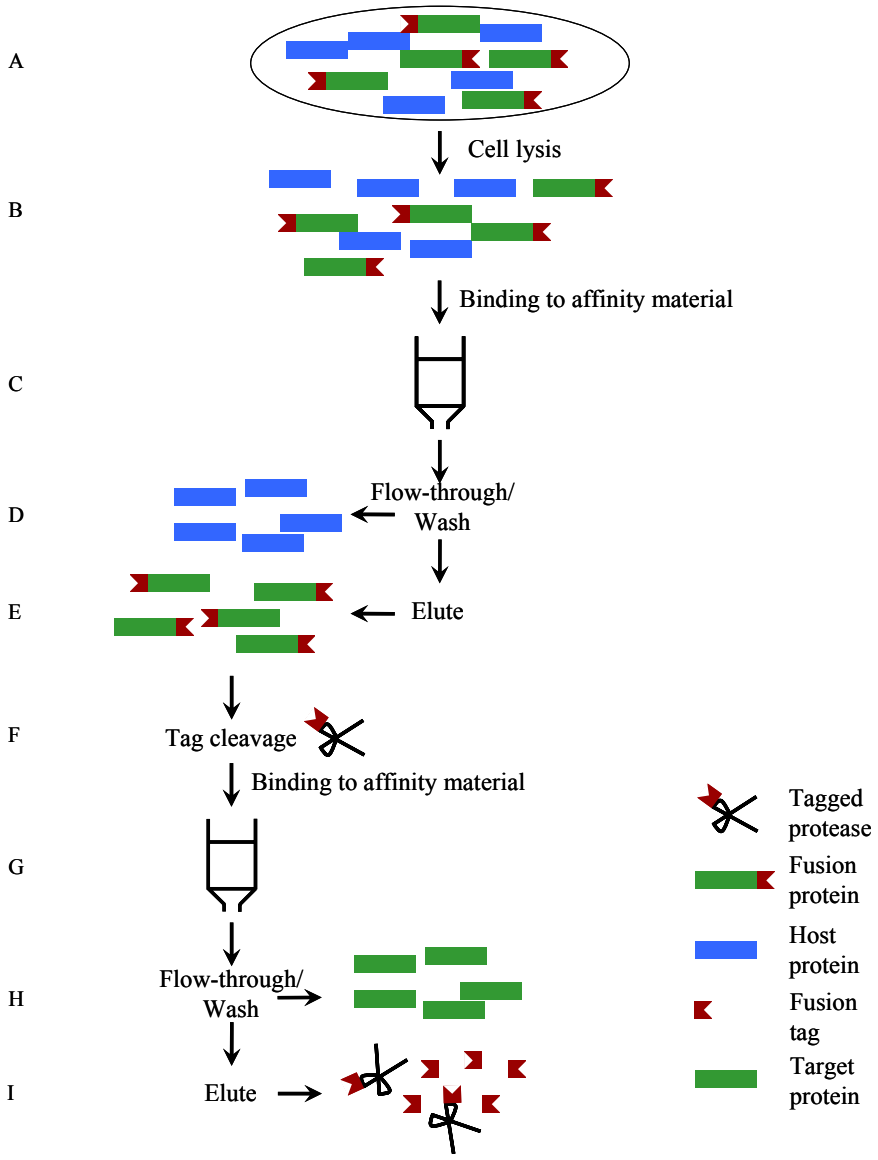


Figure 4: Overview of a typical purification scheme for a fusion (His_6- or StrepII-tagged) protein. After recombinant protein production and cell disruption **(A),** cell-free extract **(B)** is passed over the chromatographic support material with the immobilised ligand **(C)**. Ideally, only the fusion protein is bound to the material whereas other cellular host proteins remain in the flow-through and the washing steps **(D)**. The fusion protein is eluted with a high concentration of competitive ligand and apparently pure fusion protein is obtained **(E)**. After tag cleavage with a tagged protease **(F),** the reaction solution is passed over a second chromatographic column **(G)**. Only the tagged protease and the cleaved tag are bound whereas the pure target protein remains in the flow-through and the washing steps **(H)**. The protease and the tag are eluted with the competitive ligand **(I)**.

I.4.2 Codon Usage and Protein Production

The codon bias of heterologous genes is often a limiting factor for the expression in certain host system. Hence, the so called codon adaptation index (CAI) of a gene for the host is of importance. The CAI permits the calculation of a comparable value for the codon usage. The calculation of this value requires the definition of a set of highly expressed genes of an individual organism (Carbone *et al.*, 2003). The codon usage of all its other genes is calculated with respect to this subset of genes (Grote *et al.*, 2005).

An optimal adapted gene shows a CAI value of 1.0. In studies done in our group, genes encoding the hydrolase TfH of *Thermobifida fusca* (Yang *et al.*, 2006), the formiate dehydrogenase Fdh of *Mycobacterium vaccae* (Roth, 2005), and a human ceratin binding domain KbdB (Gamer, 2005; personal communication) were expressed before and after adaptation of their codon usages to *B. megaterium*. All of the listed native genes showed CAI values below 0.3. No or only a small amount of recombinant gene product was obtained. This phenomenon was previously described for *E. coli* (Jia and Li, 2005). The design and synthesis (GENEART AG; Regensburg; Germany) of the artificial genes with a CAI > 0.9 for *B. megaterium* resulted in a good production of all recombinant gene products in *B. megaterium*.

I.4.3 Model Proteins

I.4.3.1 Intracellular Model Protein: Green Fluorescent Protein from *Aequorea victoria*

For improving promoter strength or for protein production analysis, reporter or model proteins like the β-galactosidase (LacZ) from *E. coli* or the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Shimomura *et al.*, 1962; Veening *et al.*, 2004) are commonly used. Model proteins are easy to detect and show low detection limits.

GFP was discovered by Shimomura *et al.* (1962) as a companion of aequorin, the famous chemiluminescent protein from the *Aequorea* jellyfish. GFP has a relative molecular mass of 27,000. Wild type GFP absorbs light at an excitation maximum of 395 nm and fluoresces with an emission maximum of 510 nm. Beside the major excitation peak, a minor peak at 470 nm can be monitored (**Fig. 5a**). This absorption peak also permits excitation of GFP with the 488 nm line of an argon laser which is used in fluorescence activated cell sorting (FACS). Since fluorescence occurs in the absence of any substrates or cofactors, GFP is extremely useful as a model protein in a wide variety of organisms (Heim *et al.*, 1994). In later studies, variants of GFP were used for quantitative analysis of protein production (Reischer *et al.*, 2004; Scholz *et al.*, 2000). The wild type chromophore which is responsible for the absorption

of blue and the emission of green light is formed by the amino acid residues 65 to 67 (Ser-Tyr-Gly). Cormack *et al.* (1996) constructed a library of mutant *gfp* genes with substitutions in the twenty amino acids sequence flanking the chromophore. A S65T single mutation led to a 6-fold increase of fluorescence intensity (Heim *et al.*, 1994). The F64L / S65T double mutation resulted in a 30-times higher fluorescence intensity than the wild type GFP (Cormack *et al.*, 1996). This double mutation further led to a large shift in excitation maxima from 395 nm to 488 nm compared to native GFP (**Fig. 5b**). Furthermore, its fluorescence is detectable within 8 min compared to 1 – 2 h for the wild type. Accordingly, in this study the later GFP variant called enhanced GFP (EGFP or GFPmut1) was used.

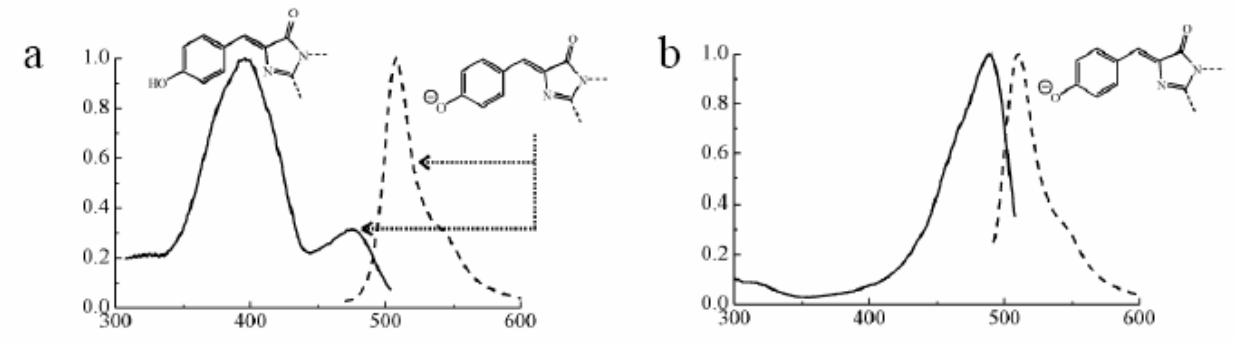


Figure 5: Fluorescence excitation and emission spectra for wild type GFP and enhanced GFP (Tsien, 1998). Fluorescence excitation (solid lines) and emission (dashed lines) spectra together with the chromophore structures believed to be responsible for the achieved spectra are shown. The spectra are normalised to a maximum amplitude of 1.0. **(a)** Spectra of wild type GFP. **(b)** Spectra of enhanced GFP (EGFP or GFPmut1).

I.4.3.2 Exported Model Protein I: Levansucrase from *Lactobacillus reuteri* Strain 121

Bacterial fructosyltransferase enzymes are found in Gram negative as well as in Gram positive bacteria. Fructosyltransferases cleave the glycosidic bond of their substrate sucrose and use the released energy to couple a fructose unit to a growing fructan chain (transfructosylation), to sucrose or to water (hydrolysis) (van Hijum *et al.*, 2006). One member of this enzyme family is the $CaCl_2$ dependent levansucrase (E.C. 2.4.1.10) from *Lactobacillus reuteri* strain 121. Levansucrases produce a β(2→6)-linked fructosyl polymer called levan. Besides polymer formation (**Fig. 6A+B**), the *L. reuteri* levansucrase also shows a high hydrolytic activity by using water as acceptor molecule (**Fig. 6C**) (van Hijum *et al.*, 2001). Polysaccharides and oligosaccharides are of high interest due to their antitumor, antiucler, immunomodulating or cholesterol-lowering activity (summarised in Varki, 1993). Levan produced by *L. reuteri* levansucrase may be a useful prebiotic due to its capacity of

β-linked fructose units which pass the gastro-intestinal tract undigested. In the colon, levan selectively stimulates the growth of beneficial gut bacteria, like *Bifidobacteria* or *Lactobacilli* (Casas *et al.*, 1988; Salminen *et al.*, 1996). Additionally, fructans like levan are employed in food industry as low caloric, noncariogenic substances and non-food industry as viscosifier, stabiliser, emulsifier, gelling, or water binding agent.

The gene *lev* encoding the levansucrase from *L. reuteri* strain 121 has previously been isolated and was expressed in *E. coli* (van Hijum *et al.*, 2004). Purified levansucrase showed an apparent M_r of 110,000 on SDS-PAGE and of 90,000 by gel filtration (van Hijum *et al.*, 2001).

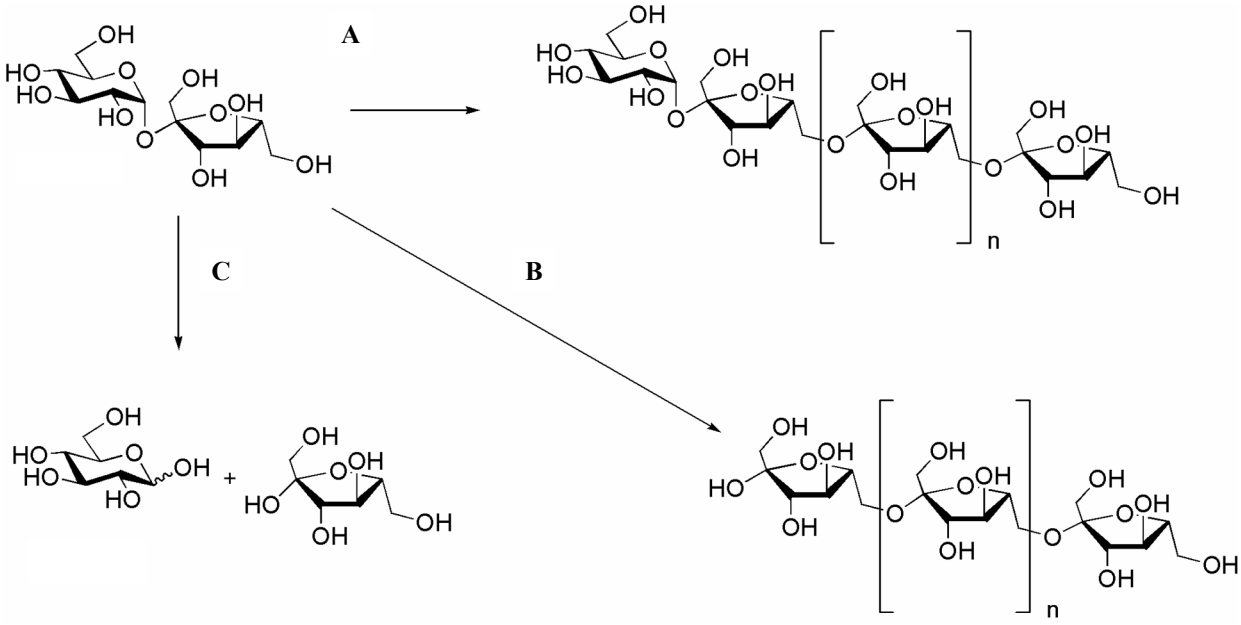


Figure 6: Reactions which are catalysed by the levansucrase from *L. reuteri* strain 121. Lev cleaves the glycosidic bond of sucrose and uses the released energy to couple a fructose unit to **(A)** a growing levan chain (transfructosylation) of n fructosyl residues starting from the first acceptor sucrose or **(B)** fructose or to **(C)** water (hydrolysis) and release of glucose and fructose.

I.4.3.3 Exported Model Protein II: Penicillin G Acylase from *Bacillus megaterium* ATCC14945

Penicillin acylases (penicillin amidases or benzyl penicillin amidohydrolases, E.C. 3.5.1.11) are found in bacteria, actinomycetes, yeasts and fungi (Mahajan, 1984). They form a group of enzymes which catalyse the cleavage of penicillin derivatives to 6-aminopenicillanic acid (6-APA) and an organic acid (**Fig. 7A**). In a reverse reaction, penicillin acylases are able to catalyse the condensation of D-amino acid derivatives containing a β-lactam (**Fig. 7C**) to synthesise semisynthetic penicillins and cephalosporines (Vandamme and Voets, 1974). In

nature, penicillin acylases with different substrate specificities are found (**Fig. 7B**). The penicillin G acylase PGA from *E. coli* is well characterised. PGA hydrolyses penicillin G to 6-aminopenicillanic acid (6-APA) and phenylacetic acid (PAA).

The penicillin G acylase of *B. megaterium* ATCC14945 is an extracellular enzyme. It is synthesised as a 92-kDa precursor molecule in the cell. After its export, it is cleaved into two unequal subunits: a 27 kDa α-subunit and a 57 kDa β-subunit (Panbangred *et al.*, 2000). The cloning and expression of the *pga* gene of *B. megaterium* in *E. coli* (Kang *et al.*, 1991; Martin *et al.*, 1995), in *B. subtilis* (Kang *et al.*, 1991; Yang *et al.*, 2001) and in *B. megaterium* itself (Panbangred *et al.*, 2000) are already described in literature. Penicillin G acylase from *B. megaterium* is one of the most common enzymes for the industrial synthesis of novel semisynthetic β-lactam antibiotics (Yang *et al.*, 2001).

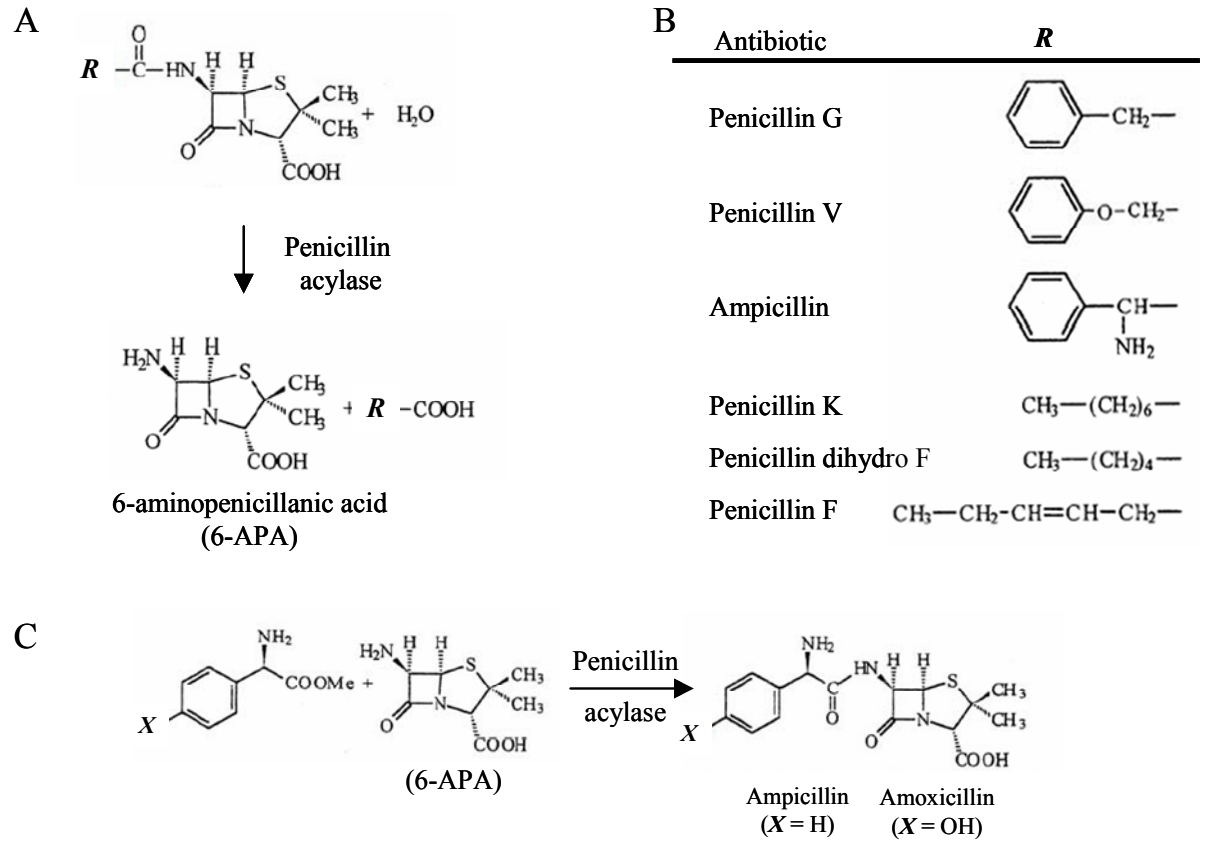


Figure 7: Substrates and reactions of penicillin acylases (modified from Arroyo *et al.*, 2003): (A) Penicillin acylase catalyses the hydrolysis of penicillins to yield 6-aminopenicillanic acid (6-APA) and the corresponding organic acid. **(B)** Side-chains of different types of penicillins. **(C)** Penicillin acylase catalyses a central step on the synthesis of some important β-lactam antibiotics. Here, the syntheses of ampicillin and amoxicillin are shown.

I.5 GENOMES

I.5.1 Genome Sequences of *Bacilli*

Availability of complete genome sequences of bacterial species significantly simplifies the investigation of these organisms. The complete DNA sequences of seven different *Bacilli* genomes have been determined so far. The genome sequence of *B. subtilis* as the most intensively studied Gram positive bacteria was published first (Kunst *et al.*, 1997). Since the year 2000, the genomes of *Bacillus halodurans* (Takami *et al.*, 2000), *Bacillus cereus* (Ivanova *et al.*, 2003), *B. anthracis* (Read *et al.*, 2003), *Bacillus licheniformis* (Rey *et al.*, 2004; Veith *et al.*, 2004), *B. thuringiensis* (DOE Joint Genome Institute; Walnut Creek; USA) and *Bacillus clausii* (Duc le *et al.*, 2004) followed. The sequenced genomes showed different sizes from approximate 4.2 Mb for the genomes of *B. subtilis*, *B. licheniformis*, *B. halodurans* and *B. clausii* to about 5.2 Mb for the genomes of *B. anthracis*, *B. cereus* and *B. thuringiensis*. Related to this, the numbers of predicted open reading frames vary from around 4,200 in case of the smaller genomes to more then 5,900 for the larger genomes of the pathogenic *Bacilli* species. Much effort was also put on the comparison of these genomes (Ivanova *et al.*, 2003; Lapidus *et al.*, 2002; Rasko *et al.*, 2004; Rey *et al.*, 2004; Takami *et al.*, 2000; Veith *et al.*, 2004) to get further idea about their relation ships.

I.5.2 Genome Sequence of *Bacillus megaterium* DSM319

Within the Collaborative Research Centre SFB578, the sequencing of the plasmidless *B. megaterium* strain DSM319 was performed by the company GATC-Biotech AG (Konstanz; Germany). Annotation of the sequences has been started in a diploma thesis (Hundertmark, 2005) in our group. In parallel, the Institute of Genomic Research (TIGR Rockville; USA) has started to sequence and annotate the genome of *B. megaterium* QM B1551 (ATCC12872) which is known to contain 7 plasmids (Kieselburg *et al.*, 1984). At the moment (autumn 2006), both sequencing and annotation processes are still in progress with so far 266 and 45 sequenced contigs, respectively.

I.6 OBJECTIVES OF THE STUDY

The main objective of this study was the development of genetic tools for the recombinant intra- and extracellular protein production using the Gram positive bacterium *B. megaterium*. The recombinantly produced proteins should be subjected to an one step purification from the host. For this purpose, a series of expression vectors for the intracellular protein production driven from the xylose-inducible promoter had to be designed and constructed. These vectors should enable the easy cloning of any gene of interest. Furthermore, the constructed plasmids should allow the fusion of a target gene to the coding regions of the His_6- and StrepII-affinity tags. These newly developed tools had to be tested for the production and purification of a recombinant model protein. The green fluorescent protein (GFP) from the jellyfish *A. victoria* was chosen (section **III.1**). Additionally, the GFP production should be upscaled to high cell density cultivation processes with in parallel analysis of the intracellular GFP formation of each individual *B. megaterium* cell. Moreover, the xylose-dependent production system should be expanded into a series of secretion vectors. These constructs shall allow the signal peptide mediated export of recombinant tagged proteins into the environment of *B. megaterium* with their final purification directly from the cell free growth medium. This system had to be tested using different homologous signal peptides – one from the *B. megaterium* esterase LipA, one from the *B. megaterium* penicillin G acylase PGA and an artificial one – with the *L. reuteri* levansucrase LevΔ773 as a model protein (section **III.2**). Finally, an alternative sucrose-inducible promoter should be studied and compared to the xylose based expression system (section **III.3**).

II MATERIAL AND METHODS

II.1 INSTRUMENTS AND CHEMICALS

II.1.1 Instruments

Agarose Gel Documentation	GelDoc	Bio-Rad
Agarose Gel Electrophoresis	Agagel	Biometra
Autoclav	LVSY 50/70	Zirbus
Bioreactor	Biostat B2	B. Braun
	Gas analysis unit	Maihak
Blotting	Trans Blot apparatus (semi dry transfer cell)	Bio-Rad
Centrifuges	Centrifuge 5403	Eppendorf
	Centrifuge 5415 C	Eppendorf
	L7-65 Ultracentrifuge	Beckmann
	RC 5B Plus	Sorvall
	SpeedVac SPD 110B with Refrigerated Vapour Trap RVT400	Savant
Cross-Flow Filtration	QuixStand Benchtop Systems	GE Healthcare
Digital Camera	Cyber shot	Sony
DNA Sequencing	ABI PRISM™ 310 Genetic Analyser	Applied Biosystems
Electroporation	Gene Pulser® II with Pulse Controller Plus	Bio-Rad
FACS	FACSCalibur	Benton Dickinson
Fluoroskan Ascent Reader	Thermo	Waltham
Gradient Cycler	Tgradient	Biometra
HPLC	Controller SCL-10A	Shimadzu
	Aminex HPX-87H column	Bio-Rad
IEF chamber	PROTEAN IEF-Cell	Bio-Rad
Luminescence Spectrometer	LS50B	PerkinElmer
Lyophiliser	LYOVAC GT 2	AMSCO/FINN-AQUA
pH Determination	pH-Meter C 6840 B	Schott
Pipettes	LABMATE	Abimed HTL

Scales	BL 1500	Sartorius
	BP 61S	Sartorius
	SBA 52	Scaltec
SDS-PAGE	Mini Protean II	Bio-Rad
Shaker	Bench Top Shaker, TR	Infors AG HT
Sonication	SONOPLUS	Bandelin electronics
Spectrophotometer	Ultrospec 200	GE Healthcare
Thermocycler	Tpersonal	Biometra
Thermomixer	Thermomixer compact	Eppendorf
Water Bath Shaker	Aquatron	Infors AG HT
Water Purification	Synthesis A10	Millipore

II.1.2 Chemicals and Kits

Bio-Rad Protein Assay	Bio-Rad
Cell Viability Kit	BD Bioscience
Chelating Sepharose FF	GE Healthcare
PD-10 Column	GE Healthcare
Enzymes for Molecular Biological Applications	GE Healthcare Genecraft MBI-Fermentas New England BioLabs Promega
Factor Xa Protease	QIAgen
MagneHis™ Ni-Particles	Promega
QIAquick Gel Extraction Kit	QIAgen
QIAquick PCR Purification Kit	QIAgen
QuikChange® Site-Directed Mutagenesis Kit	Stratagene
Size Standards for Agarose Gels:	
GeneRuler™ DNA Ladder Mix	MBI Fermentas
MassRuler™ DNA Ladder Mix	MBI Fermentas

Size Standards for SDS-PAGE:	
High Molecular Weight Marker	Sigma
PageRuler Prestained Protein Ladder	MBI Fermentas
Precision Plus Protein Standard	Bio-Rad
Protein Molecular Weight Marker	MBI Fermentas
Strep-Tactin Superflow	IBA GmbH

Chemicals and reagents not specifically listed here were purchased from the following manufacturers: Difco, Fluka, GE Healthcare, Gerbu, Merck, Oxoid, Riedel-de-Häen, Roth, and Sigma-Aldrich.

II.2 BACTERIAL STRAINS AND PLASMIDS

All bacterial strains and plasmids used for this study are listed in **table 1** and **2**.

Table 1: Strains used in this study

Name	Description	Reference/source
Bacillus megaterium strains		
DSM319	Wild type	DSMZ, Braunschweig
WH320	Mutant of DSM319, *lacZ*	(Rygus *et al.*, 1991)
WH323	Mutant of WH320, *xylA1-spoVG-lacZ*	(Rygus and Hillen, 1992)
MS941	Mutant of DSM319, *ΔnprM*	(Wittchen and Meinhardt, 1995)
ATCC14945	Penicillin G acylase producer	American Type Culture Collection

Table 1 (continued): Strains used in this study

Name	Description	Reference/source
Escherichia coli strains		
DH10B	*F*⁻ mcrA *Δ(*mrr-hsd*RMS*-mcr*BC) ϕ80d*lacZ*ΔM15 Δ*lacX*74* deo*R* recA*1* end*A1* araD*139 Δ(*ara, leu*)7697* galU galK *λ*⁻ rpsL nupG	Gibco Life Technologies
XL1-Blue	recA*1* endA*1* gyrA*96* thi-*1* hsdR*17* supE*44* rel*A1* lac *[F'* proAB lacI^{q}Z*ΔM15 Tn10 (Tet^{r})]*	Stratagene

Table 2: Plasmids used in this study

Plasmid designation	Description	Reference/source
pBAD-*lev*	pBAD/MycHisC with truncated form of the levansucrase gene (*levΔ773*) from *L. reuteri* 121; *levΔ773*-Myc-His$_6$-Tag	(van Hijum *et al.*, 2001)
pMUTIN-GFP+	Integration vector designed to fuse proteins with a high-fluorescence variant of GFP	*Bacillus* Genetic Stock Centre; USA
pMM1520	Shuttle vector for cloning in *E. coli* (*Amp^{r}*) and gene expression under xylose control in *B. megaterium* (*Tet^{r}*); P$_{xylA}$-MCS	(Malten *et al.*, 2005a)
pYZ5	Truncated form of pYZ11 (Kunnimalaiyaan *et al.*, 2001), shuttle vector for cloning in *E. coli* (*Amp^{r}*) and replication in *B. megaterium* (*Cml^{r}*) containing 1.1 kb fragment of pBM100	(Vary, personal gift)
pKM704	Shuttle vector for cloning *E. coli* (*Amp^{r}*) and replication in *B. megaterium* (*Cml^{r}*) containing 6.4 kb *Eco*RI fragment of pBM700 in pJM103 (Perego *et al.*, 1988)	(Vary, personal gift)
Plasmids for production of intracellular recombinant proteins		
pMM1522	*Bsr*GI restriction site inserted into pMM1520 upstream of the start codon of the open reading frame including the MCS; P$_{xylA}$-MCS	this study
pSTOP1522	pMM1522 derivative with a stop codon directly downstream of the *Nae*I restriction site followed by additional *Nru*I and *Age*I sites; P$_{xylA}$-MCS-Stop	this study

Table 2 (continued): Plasmids used in this study

Plasmid designation	Description	Reference/source
pHIS1522	pSTOP1522 derivative; vector for the intracellular production of C-terminal His_6-tagged proteins in *B. megaterium*; P_{xylA}-MCS-His_6-Tag-Stop	this study
pSTREP1522	pSTOP1522 derivative; vector for the intracellular production of N-terminal StrepII-tagged proteins in *B. megaterium*; P_{xylA}-StrepII-Tag-Xa-MCS-Stop	this study
pSTOP1622	Identical to pSTOP1522, but lacking 855 bp between the two *Afl*II restriction sites	this study
pC-HIS1622	Identical to pHIS1522, but lacking 855 bp between the two *Afl*II restriction sites	this study
pN-STREP-Xa1622	Identical to pSTREP1522, but lacking 855 bp between the two *Afl*II restriction sites	this study
pC-STREP1622	pSTOP1622 derivative; vector for the intracellular production of C-terminal StrepII-tagged proteins in *B. megaterium*; P_{xylA}-MCS-StrepII-Tag-Stop	this study
pN-HIS-TEV1622	pSTOP1622 derivative; vector for the intracellular production of N-terminal His_6-tagged proteins in *B. megaterium*; P_{xylA}-His_6-Tag-TEV-MCS-Stop	this study
pN-STREP-TEV1622	pSTOP1622 derivative; vector for the intracellular production of N-terminal StrepII-tagged proteins in *B. megaterium*; P_{xylA}-StrepII-Tag-TEV-MCS-Stop	this study
pMGBm19	*xylR*-P_{xylA}-*xylA'*-MCS cloned into *Bam*HI and *Nar*I of pYZ5	(Gamer, personal gift)
pMGBm21	*xylR*-P_{xylA}-*xylA'*-MCS cloned into *Eco*RV and *Pst*I of pKM704	(Gamer, personal gift)
pRBBm34	*gfp* including the sequence of its stop codon cloned into *Bgl*II and *Eag*I of pMM1522 creating P_{xylA}-*gfp*-Stop	this study
pRBBm35	*gfp* cloned into *Bgl*II and *Sph*I of pC-HIS1622 creating P_{xylA}-*gfp*-His_6-Tag-Stop	this study
pRBBm36	*gfp* cloned into *Bgl*II and *Sph*I of pN-Strep-Xa1622 creating P_{xylA}-StrepII-Tag-Xa-*gfp*-Stop	this study
pRBBm53	*gfp* cloned into *Bgl*II and *Sph*I of pSTOP1622 creating P_{xylA}-*gfp*-Stop	this study

Table 2 (continued): Plasmids used in this study

Plasmid designation	Description	Reference/source
pRBBm54	*gfp* cloned into *Bgl*II and *Sph*I of pN-HIS-TEV1622 creating P_{xylA}-His_6-Tag-TEV-*gfp*-Stop	this study
pRBBm55	*gfp* cloned into *Bgl*II and *Sph*I of pN-Strep-TEV1622 creating P_{xylA}-StrepII-Tag-TEV-*gfp*-Stop	this study
pRBBm56	*gfp* cloned into *Bgl*II and *Sph*I of pC-STREP1622 creating P_{xylA}-*gfp*-StrepII-Tag-Stop	this study
pRBBm63	*gfp* cloned into *Spe*I and *Xma*I of pMGBm19 creating P_{xylA}-*gfp*-Stop	this study
pRBBm64	*gfp* cloned into *Xma*I and *Eag*I of pMGBm21 creating P_{xylA}-*gfp*-Stop	this study
pRBBm67	$trxA_{B.meg}$ cloned into *Spe*I of pRBBm55 creating P_{xylA}-*trxA*-StrepII-Tag-TEV-*gfp*-Stop	this study
pRBBm68	$trxA_{B.meg}$ cloned into *Spe*I of pRBBm54 creating P_{xylA}-*trxA*-His_6-Tag-TEV-*gfp*-Stop	this study
pRBBm40	*repU-oriU* of pMM1520 cloned into the *Aat*II site of pMUTIN-gfp+	this study
pRBBm59	676 bp of promoter $P_{sacBB.meg}$ including the first 39 bp of *sacB* from *B. megaterium* cloned into *Afl*II and *Spe*I of pRBBm34 creating $P_{sacBB.meg}$-$sacB'_{B.meg}$-*gfp*	this study
pRBBm70	316 bp of promoter $P_{sacBB.meg}$ including the first 39 bp of *sacB* from *B. megaterium* cloned into *Afl*II and *Spe*I of pRBBm34 creating $P_{sacBB.meg}$I-$sacB'_{B.meg}$-*gfp*	this study
pRBBm71	221 bp of promoter $P_{sacBB.meg}$ including the first 39 bp of *sacB* from *B. megaterium* cloned into *Afl*II and *Spe*I of pRBBm34 creating $P_{sacBB.meg}$II-$sacB'_{B.meg}$-*gfp*	this study
pRBBm72	122 bp of promoter $P_{sacBB.meg}$ including the first 39 bp of *sacB* from *B. megaterium* cloned into *Afl*II and *Spe*I of pRBBm34 creating $P_{sacBB.meg}$III-$sacB'_{B.meg}$-*gfp*	this study
Plasmids for production of extracellular recombinant proteins		
pMM1525	pMM1522 derivative containing the DNA sequence coding for the signal peptide of the *B. megaterium* extracellular esterase LipA (SP_{lipA}) between the *Bsr*GI and *Bst*BI sites; P_{xylA}-SP_{lipA}-MCS	this study
pMM1533	pMM1525 with additional *Bst*BI site downstream of sp_{lipA}; P_{xylA}-SP_{lipA}-MCS	this study

Table 2 (continued): Plasmids used in this study

Plasmid designation	Description	Reference/source
pADBm5	pMM1533 derivative without *Sfo*I site downstream of sp_{lipA}; P_{xylA}-SP_{lipA}-MCS	(Drews, 2004)
pRBBm26	pSTREP1522 derivative containing the DNA sequence coding for the signal peptide of *B. megaterium* ATCC14945 penicillin G acylase PGA (SP_{pga}) between the *Bsr*GI and *Spe*I sites; P_{xylA}-SP_{pga}-StrepII-Tag-Xa-MCS	this study
pRBBm27	pSTREP1522 derivative containing the DNA sequence coding for the signal peptide of *B. megaterium* ATCC14945 penicillin G acylase PGA (SP_{pga}) and part of the α-subunit between the *Bsr*GI and *Spe*I sites; P_{xylA}-SP_{pga}-*α-subunit*''-StrepII-Tag-Xa-MCS	this study
pRBBm28	pSTREP1522 derivative containing the DNA sequence coding for the signal peptide of *B. megaterium* ATCC14945 penicillin G acylase PGA (SP_{pga}) and part of the α-subunit between the *Bsr*GI and *Spe*I sites; P_{xylA}-SP_{pga}-*α-subunit*'-StrepII-Tag-Xa-MCS	this study
pRBBm29	pSTREP1522 derivative containing the DNA sequence coding for the signal peptide of *B. megaterium* ATCC14945 penicillin G acylase PGA (SP_{pga}) and the α-subunit between the *Bsr*GI and *Spe*I sites; P_{xylA}-SP_{pga}-*α-subunit*-StrepII-Tag-Xa-MCS	this study
pADBm20	pMM1522 derivative containing the DNA sequence coding for an artificial signal peptide (SP_{asp}) between *Sac*I and *Bgl*II; P_{xylA}-SP_{asp}-MCS	(Drews, 2004)
pSTREP1525	pMM1525 derivative; vector for the secretion of recombinant N-terminal StrepII-tagged proteins in *B. megaterium*; P_{xylA}-SP_{lipA}-StrepII-Tag-Xa-MCS-Stop	this study
pHIS1525	pADBm5 derivative; vector for the secretion of recombinant C-terminal His_6-tagged proteins in *B. megaterium*; P_{xylA}-SP_{lipA}-MCS-His_6-Tag-Stop	this study
pSTREPHIS1525	pMM1525 derivative; vector for the secretion of recombinant N-terminal StrepII-tagged and C-terminal His_6-tagged proteins in *B. megaterium*; P_{xylA}-SP_{lipA}-StrepII-Tag-Xa-MCS-His_6-Tag-Stop	this study

Table 2 (continued): Plasmids used in this study

Plasmid designation	Description	Reference/source
pRBBm23	Penicillin G acylase gene *pga* of *B. megaterium* ATCC14945 including the sequence of its signal peptide SP_{pga} cloned into *Bsr*GI and *Sac*I of pMM1522 creating P_{xylA}-SP_{pga}-*pga*	this study
pRBBm24	*levΔ773*MycHis cloned into *Bgl*II and *Sph*I of pADBm20 creating P_{xylA}-SP_{asp}-*levΔ773*-Myc-His_6-Tag	this study
pMMBm6	*levΔ773*MycHis cloned into *Sac*I and *Sph*I of pMM1525 with linker creating P_{xylA}-SP_{lipA}-linker-*levΔ773*-Myc-His_6-Tag	this study
pMMBm7	pMMBm6 after elimination of the linker region creating P_{xylA}-SP_{lipA}-*levΔ773*-Myc-His_6-Tag	
pMGBm4	pMMBm7 derivative after site-directed mutagenesis creating P_{xylA}-SP_{lipA}-*levΔ773*-Stop	this study
pRBBm13	*levΔ773* cloned into *Bgl*II and *Sph*I of pSTREP1525 creating P_{xylA}-SP_{lipA}-StrepII-Tag-Xa-*levΔ773*	this study
pRBBm14	*levΔ773* cloned into *Sac*I and *Sph*I of pHIS1525 creating P_{xylA}-SP_{lipA}-linker-*levΔ773*-His_6-Tag	this study
pRBBm15	pRBBm14 after elimination of the linker region creating P_{xylA}-SP_{lipA}-*levΔ773*-His_6-Tag	this study
pRBBm16	*levΔ773* cloned into *Bgl*II and *Sph*I of pSTREPHIS1525 creating P_{xylA}-SP_{lipA}-StrepII-Tag-Xa-*levΔ773*-His_6-Tag	this study
pEJBm2	*strepII* and terminator cloned into *Sph*I and *Age*I of pRBBm15 creating P_{xylA}-SP_{lipA}-*levΔ773*-StrepII-Tag-terminator	(Jordan, personal gift)
pEJBm7	his_6 and terminator cloned into *Sph*I and *Age*I of pRBBm15 creating P_{xylA}-SP_{lipA}-*levΔ773*-His_6-Tag-terminator	(Jordan, personal gift)
pRBBm30	*levΔ773* cloned into *Bgl*II and *Sph*I of pRBBm26 creating P_{xylA}-SP_{pga}-StrepII-Tag-Xa-*levΔ773*	this study
pRBBm31	*levΔ773* cloned into *Bgl*II and *Sph*I of pRBBm27 creating P_{xylA}-SP_{pga}-*α-subunit''*-StrepII-Tag-Xa-*levΔ773*	this study

Table 2 (continued): Plasmids used in this study

Plasmid designation	Description	Reference/source
pRBBm32	*levΔ773* cloned in *Bgl*II and *Sph*I of pRBBm28 creating P_{xylA}-SP_{pga}-*α-subunit'*-StrepII-Tag-Xa-lev*Δ773*	this study
pRBBm33	*levΔ773* cloned in *Bgl*II and *Sph*I of pRBBm29 creating P_{xylA}-SP_{pga}-*α-subunit*-StrepII-Tag-Xa-*levΔ773*	this study
pMMBm3	Vector for coexpression of *dsrS* under the control of P_{xylA} and $sipM_{B.meg}$ under control of P_{sipM}; P_{sipM}-*sipM* -*xylR*-P_{xylA}-*dsrS*	(Malten *et al.*, 2005b)
pMMBm9	Vector for expression of $prsA_{B.sub}$ under control of P_{prsA}; P_{prsA}-*prsA*	(Malten, 2005)
pRBBm17	*Aat*II/*Xho*I fragment containing P_{sipM}-*sipM* subcloned from pMMBm3 to *Xho*I/*Aat*II fragment of pRBBm23; P_{sipM}-*sipM-xylR*-P_{xylA}-SP_{pga}-*pga*	this study
pRBBm18	*Sac*I/*Xho*I fragment containing P_{prsA}-*prsA* subcloned from pMMBm9 to *Xho*I/*Sac*I fragment of pRBBm23; P_{prsA}-*prsA-xylR*-P_{xylA}-SP_{pga}-*pga*	this study
pRBBm19	*Sph*I/*Xho*I fragment containing P_{sipM}-*sipM* subcloned from pMMBm3 to *Xho*I/*Sph*I fragment of pMMBm7; P_{sipM}-*sipM-xylR*-P_{xylA}-SP_{lipA}-*levΔ773*-Myc-His_6-Tag	this study
pRBBm20	*Sph*I/*Xho*I fragment containing P_{prsA}-*prsA* subcloned from pMMBm9 to *Xho*I/*Sph*I fragment of pMMBm7; P_{prsA}-*prsA-xylR*-P_{xylA}-SP_{lipA}-*levΔ773*-Myc-His_6-Tag	this study
pRBBm46	*Sph*I/*Xho*I fragment containing P_{sipM}-*sipM* subcloned from pMMBm3 to *Xho*I/*Sph*I fragment of pEJBm2; P_{sipM}-*sipM-xylR*-P_{xylA}-SP_{lipA}-*levΔ773*-His_6-Tag-terminator	this study
pRBBm69	$P_{sacBB.meg}$-$sacB_{B.meg}$ from *B. megaterium* cloned into *Afl*II and *Bam*HI of pMM1522	this study

II.3 GROWTH MEDIA AND MEDIA ADDITIVES

II.3.1 Media for *Escherichia coli* and *Bacillus megaterium* Growth

As a standard medium for growth of all bacterial strains, Luria Bertani (LB) medium (Sambrook *et al.*, 1999) was used unless indicated otherwise. For solid media, 1.5 % (w/v) agar-agar was added before sterilisation.

LB medium	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	H_2O_{deion}	ad 1.0 l

For the recombinant protein production, *B. megaterium* plasmid strains were grown in LB medium, TB medium, A5 medium (Malten *et al.*, 2005a), A5+4 medium or A5 MOPSO medium.

TB medium	Tryptone	12.0 g
	Yeast extract	24.0 g
	Glycerol	4.0 g
	Casamino acids	1.0 g
	H_2O_{deion}	ad 880.0 ml
after autoclaving, addition of:	KH_2PO_4/K_2HPO_4 (170 mM/720 mM)	100.0 ml
	Glucose (1 M)	20.0 ml
	Thiamine chloride hydrochloride (50 µg ml^{-1})	1.0 ml

A5 medium (Malten *et al.*, 2005a)	$(NH_4)_2SO_4$	2.0 g
	$MgSO_4$ x 7 H_2O	0.3 g
	Yeast extract	0.5 g
	Trace element solution (1000 x)	1.0 ml
	H_2O_{deion}	ad 800.0 ml
after autoclaving, addition of:	Glucose 30 % (w/v)	100.0 ml
	10 x Phosphate buffer	100.0 ml

A5+4 medium	$(NH_4)_2SO_4$	2.0 g
	$MgSO_4$ x 7 H_2O	0.3 g
	Yeast extract	4.0 g
	Trace element solution (1000 x)	1.0 ml
	H_2O_{deion}	ad 800.0 ml
after autoclaving, addition of:	Glucose 30 % (w/v)	100.0 ml
	10 x Phosphate buffer	100.0 ml

A5 MOPSO medium	NaCl	2.92 g
	KCl	0.75 g
	$(NH_4)_2SO_4$	2.0 g
	$MgSO_4$ x 7 H_2O	0.3 g
	Yeast extract	4.0 g
	Trace element solution (1000 x)	1.0 ml
	H_2O_{deion}	ad 800.0 ml
after autoclaving, addition of:	Glucose 30 % (w/v)	100.0 ml
	10 x MOPSO buffer	100.0 ml
	K_2HPO_4 (1.32 M)	1.0 ml

Trace element solution (1000 x)	$MnCl_2$ x 4 H_2O	40.0 g
	$CaCl_2$ x 2 H_2O	53.0 g
	$FeSO_4$ x 7 H_2O	2.5 g
	$(NH_4)_6Mo_7O_{24}$ x 4 H_2O	2.5 g
	$CoCl_2$ x 6 H_2O	2.5 g
	H_2O_{deion}	ad 1.0 l
10 x Phosphate buffer	KH_2PO_4	35.2 g
	Na_2HPO_4 x 2 H_2O	72.6 g
	H_2O_{deion}	ad 1.0 l
10 x MOPSO buffer (pH 6.8)	MOPSO 50 mM	112.64 g
	H_2O_{deion}	ad 1.0 l
	titrated with KOH to pH 6.8	

II.3.2 Additives

Antibiotics and other additives were prepared as concentrated stock solutions, sterilised by filtration and added after autoclaving to the chilled medium. For light sensitive additives such as tetracycline, incubation occurred in the dark. Solutes and concentrations are summarised in **table 3**.

Table 3: Additives

Substances	Solute	Concentration of stock solution	Final concentration in growth medium
Carbenicillin	H_2O_{deion}	100 mg ml^{-1}	100 μg ml^{-1}
Tetracycline	Ethanol 70 % (v/v)	5 mg ml^{-1}	10 μg ml^{-1}
IPTG	H_2O_{deion}	1 M	500 μM
Xylose	H_2O_{deion}	20 % and 50 % (w/v)	0.01 – 2 % (w/v)
Sucrose	H_2O_{deion}	50 % (w/v)	0.01 – 1 % (w/v)
Chloramphenicol	Ethanol 70 % (v/v)	34 mg ml^{-1}	4.5 μg ml^{-1}
K_2HPO_4	H_2O_{deion}	1.32 M	0.16 – 1.32 mM
Erythromycin	Ethanol 70 % (v/v)	10 mg ml^{-1}	0.5 – 5 μg ml^{-1}
$CaCl_2$	H_2O_{deion}	1 M	2.5 mM

II.4 MICROBIOLOGICAL TECHNIQUES

II.4.1 Sterilisation

Unless noted otherwise, all media were vapour sterilised at 121°C and 1 bar positive pressure for 20 min. Other substances and solutions were either vapour sterilised or, if temperature sensitive, sterilised by filtration (pore width of the filter was 0.2 μm).

II.4.2 Plate Cultures

Bacteria were plated directly from a glycerol stock onto a LB medium agar plate. When required, antibiotics or other additives were added. Agar plates were incubated overnight at 30°C or 37°C.

II.4.3 Liquid Cultures of *Escherichia coli*

Aerobic liquid cultures were inoculated using a single colony from a LB medium agar plate. The medium was supplemented with the appropriate antibiotics when required. Cultures were shaken at 200 rpm in test tubes or baffled flasks at 37°C. The incubation times varied according to the desired optical densities.

II.4.4 Liquid Cultures of *Bacillus megaterium*

Aerobic liquid cultures were inoculated using a single colony from a LB medium agar plate for pre-cultures. The medium was supplemented with the appropriate antibiotics when required. Incubation occurred at 37°C in baffled flasks at 100 rpm for 14 h or at 250 rpm for 8 h.

Aerobic liquid main cultures were inoculated at the ratio of 1 : 50 to 1 : 100 from the pre-cultures. The culture volume varied from 50 to 500 ml in baffled flasks at 250 rpm and 30°C or 37°C. The incubation times varied according to the desired optical densities.

II.4.5 Determination of Cell Density

The cell densities of liquid cultures were determined by measuring the optical density at a wavelength of 578 nm (OD_{578nm}). For cell densities with an $OD_{578nm} > 0.8$, dilutions of the cell culture broth were prepared before the measurement. An OD_{578nm} of 1 corresponded to approximately 1×10^9 cells per ml.

In batch cultivations of the corresponding *B. megaterium* strains, a correlation between OD_{578nm} and cell dry weight (CDW) was determined: $OD_{578nm} \times 0.346$ = CDW (g WH323 l^{-1}), $OD_{578nm} \times 0.320$ = CDW (g WH320 l^{-1}) and $OD_{578nm} \times 0.334$ = CDW (g MS941 l^{-1}).

II.4.6 Storage of Bacteria

Strains were kept on LB medium agar plates at 4°C for up to 10 days (*B. megaterium*) or up to 4 weeks (*E. coli*). For long-term storage of bacteria, glycerol cultures were used. Therefore, *B. megaterium* cultures were incubated for 14 h and 100 rpm at 37°C. A culture volume of 650 µl was gently mixed with 350 µl of 87 % (w/v) glycerol (final concentration of 30 % (w/v) glycerol). Stocks were frozen and stored at -80°C.

II.5 MOLECULAR BIOLOGY TECHNIQUES

Protocols employed in this study are generally based on the methods described by Sambrook *et al.* (2001). Modifications of these denoted methods will be described below.

II.5.1 Preparation of Genomic and Total DNA from *Bacillus megaterium*

Small amounts of genomic DNA (up to 35 µg) from *B. megaterium* were used for amplifying genomic fragments by PCR (see II.5.5). Therefore, 4 x 10^9 cells of an overnight culture were harvested by centrifugation (14,000 x g; 15 min; 4°C) and suspended in 50 µl of lysozyme solution (10 µg ml^{-1} of lysozyme in 100 mM of sodium phosphate buffer, pH 7.0). Incubation occurred at 37°C and 1,000 rpm (Thermomixer; Eppendorf; Germany) for 1 h. In an interval of 10 min, the cell suspension was intensively mixed to advance cell lysis. After an additional incubation for 15 min at 99°C, the cell debris was separated from the DNA by centrifugation (12,000 x g; 10 min; 4°C). Before use, the DNA-supernatant was diluted 1 : 10.

II.5.2 Preparation of Plasmid DNA from *Escherichia coli*

High quality plasmid DNA in small quantities (50 – 100 µg of DNA) for protoplast transformation of *B. megaterium* was prepared from *E. coli* DH10B carrying the corresponding plasmid using the mini Prep Kit from QIAgen (QIAgen; Hilden; Germany) according to the manufacturer's instructions. H_2O_{deion} was used for elution of the DNA from the columns.

Alternative, mini-preparation of plasmid DNA from *E. coli* for other applications was carried out as following. Cells containing the desired plasmid of a 5 ml overnight culture were harvested (14,000 x g; 2 min). The cells were suspended in 300 µl of buffer P1. After addition of 300 µl of buffer P2, the tube was inverted 6 times and incubated for 2 min. Then 300 µl of buffer P3 were added and mixed carefully by 6 times inverting of the tube. Afterwards, the samples were centrifuged for 30 min at 14,000 x g. The supernatant was gently mixed with 600 µl isopropanol to precipitate the plasmid DNA followed by another centrifugation step for 30 min. Recovered DNA was washed with 400 µl of 70 % (v/v) ethanol. Finally, the DNA precipitate was dried and dissolved in 50 µl of H_2O_{deion}.

All steps occurred at RT.

II.5.2.1 Solution for Plasmid DNA Preparation

Buffer P1	Tris-HCl (pH 8.0)	50.0	mM
	EDTA	10.0	mM
	RNase A	100.0	mg l^{-1}
	dissolved in H_2O_{deion}		
Buffer P2	NaOH	200.0	mM
	SDS	1.0	% (w/v)
	dissolved in H_2O_{deion}		
Buffer P3 (pH 5.5)	CH_3COOK	3.0	M
	dissolved in H_2O_{deion}		

II.5.3 Determination of DNA Concentration

For determination of the prepared plasmid DNA concentration, the plasmid was enzymatically linearised and visualised on an agarose gel (see II.5.4). Using the software for gel documentation (Quantity One; Bio-Rad; Munich; Germany), the concentration of the respective band in the agarose gel was determined in comparison to two bands of known concentration.

The concentration and purity of prepared genomic DNA was determined by measuring the absorbance at 260 nm and additionally at 280 nm to account for protein impurities. For a pure DNA solution, an A_{260nm} of 1 corresponded to a concentration of dsDNA of 50 µg ml^{-1}.

The quality of the DNA solution is deduced from the ratio of A_{260nm} to A_{280nm}. With A_{260nm}/A_{280nm} = 1.8 – 2.0, the DNA can be considered as pure.

II.5.4 Agarose Gel Electrophoresis

For analytical separation of DNA fragments, agarose gels consisting of 0.7 to 2.5 % (w/v) agarose (according to the expected fragment size) in TAE buffer were prepared. The DNA samples were mixed with 6 x DNA loading dye to facilitate loading and to indicate the progress of the samples in the gel. GeneRuler™ DNA Ladder Mix or MassRuler™ DNA Ladder Mix (MBI Fermentas; St. Leon-Rot; Germany) were used as size standards according to the manufacturer's instructions. Depending on the size of the gel, a voltage of 80 – 100 V was applied. The DNA fragments migrate towards the anode with a velocity that is proportional to the negative logarithm of their length. After electrophoresis, gels were

incubated in an ethidium bromide solution for 30 min and briefly rinsed with H_2O_{deion}. The DNA was detected via its fluorescence under UV light (λ = 312 nm).

II.5.4.1 Solutions and Marker for Agarose Gel Electrophoresis

TAE buffer (pH 8.0)	Tris-acetate	40.0	mM
	EDTA	1.0	mM
	dissolved in H_2O_{deion}		
6 x DNA loading dye	Bromophenol blue	350.0	µM
	Xylene cyanol FF	450.0	µM
	Glycerol	50.0	% (w/v)
	dissolved in H_2O_{deion}		
Ethidium bromide solution	Ethidium bromide	0.1	% (w/v)
	dissolved in H_2O_{deion}		

GeneRuler DNA Ladder Mix (MBI Fermentas; St. Leon-Rot; Germany)

The marker contains the following fragments (given in base pairs):

10,000; 8,000; 6,000; 5,000; 4,000; 3,500; 3,000; 2,500; 2,000; 1,500; 1,200; 1,031; 900; 800; 700; 600; 500; 400; 300; 200; 100.

MassRuler DNA Ladder Mix (MBI Fermentas; St. Leon-Rot; Germany)

The marker contains the following fragments (given in base pairs):

10,000; 8,000; 6,000; 5,000; 4,000; 3,000; 2,500; 2,000; 1,500; 1,031; 900; 800; 700; 600; 500; 400; 300; 200; 100; 80.

II.5.5 Amplification of DNA by Polymerase Chain Reaction (PCR)

For amplification of DNA by PCR, oligonucleotide primers for each DNA fragment of interest were designed. Recognition sequences for restriction endonucleases were inserted via these primers at both ends of the corresponding fragment. All oligonucleotide primers are listed in the **table 4.** Recognition sequences of restriction endonucleases are *italicised*. Primers were purchased from MWG Biotech AG (Ebersberg; Germany) or biomers.net GmbH (Ulm; Germany).

Table 4: Oligonucleotide primers used for PCR. Recognition sequences of restriction endonucleases are *italicised*.

Primer designation	Primer sequence (5' – 3')
XylR_for_YZ5	GATAT*GGCGCC*CTTTGCGTTCACTTAACTAACTTATAGG
MCS_rev_YZ5	CATAT*GGATCC*GTTTGCGCATTCACAGTTCTCC
XylR_for_KM704	GATAT*GATATC*CTTTGCGTTCACTTAACTAACTTATAGG
MCS_rev_KM704	CATAT*CTGCAG*GTTTGCGCATTCACAGTTCTCC
GFP+for	TTATT*AGATCT*GGGCTAGCAAAGGAGAAGAAC
GFP+rev	ATAT*CGGCCG*CGAATTCATTATTTGTAGAGC
GFP+revII	AATAT*GCATGC*AATTTGTAGAGCTCATCCATGCC
GFP_for_XmaI	TTATT*CCCGGG*GCTAGCAAAGGAGAAGAAC
GFP_rev_EagI	AATAT*CGGCCG*TTATTTGTAGAGCTCATCC
GFP_for_SpeI	TTATT*ACTAGT*GCTAGCAAAGGAGAAGAAC
GFP_rev_XmaI	AATAT*CCCGGG*TTATTTGTAGAGCTCATCC
RepU1520_for	ATCGT*GACGTC*ACTTATACAACAATATGG
RepU1520_rev	ATCGT*GACGTC*TAACTCATAACCGAGAGG
Thio-GFP_for	ATTCG*ACTAGT*ATGGCTATTGCACATGCTACAG
Thio-GFP_rev	ATGTA*ACTAGT*CGCATGTTTATTAATTAATTCTGTTAATGC
PsacB_for	TATCA*CTTAAG*CTGATTCCAGCCGTGAAGG
PsacB_forI	TATCA*CTTAAG*GCAACGTCTGGAAATCGTGG
PsacB_forII	TATCA*CTTAAG*GTCCTAGTTACAGCTCAATG
PsacB_forIII	TATCA*CTTAAG*GAACCGGTTCTGTTTTCGG
PsacB_rev	TTATT*ACTAGT*AGCAGCAGTTGTATGCTTAGC
pRBEc1_rev	TATCA*GGATCC*GCTATTGCAAAGCGCTCAGTC
LevΔ773_for	TTATT*GAGCTC*T*GGCGCC*GATCAAGTAGAAAGTAACAATTACAACG
LevΔ773_rev	CAAGA*GCATGC*TGAAAATCTTCTCTCATC
LevΔ773_forI	TTATT*AGATCT*CCGATCAAGTAGAAAGTAACAATTACAACG

Table 4 (continued): Oligonucleotide primers used for PCR. Recognition sequences of restriction endonucleases are *italicised*.

Primer designation	Primer sequence (5' – 3')
LevΔ773_revI	CAAGA*GCATGC*AATTGTTTTGCATCGGTATTCTTACC
Sp_pga_for	CCGACTAGACCATAAGGG
Sp_pga_rev	CCTTGACCCCTTC*ACTAGT*ATCCTCCCCAGC
Sp_pgaI_rev	CTTTATT*ACTAGT*TCCATCTCTTCTGGATTGC
Sp_pgaII_rev	CAATGCTTGTAGGAGC*ACTAGT*ATCATTTTTCCACAC
Sp_pgaIII_rev	CCGACTATGGCGGC*ACTAGT*GCCTATCTTTAACGG
Oligo_pga_for	TACATA*TGTACA*ATGAAGACGAAGTGGCTAATATCA
Oligo_pga_rev	TATC*AGAGCTC*ATCAATAGTATAGGCTCTTTATGC

For amplification of the DNA of interest, PCR reactions of a total volume of 20 µl were prepared. For amplification of DNA fragments larger than 1,000 bp, the Phusion™ polymerase (Finnzymes; Espoo; Finland) whereas for amplification of smaller fragments the *Taq* polymerase (BioTherm®; genecraft; Lüdinghausen; Germany) was used. The Phusion polymerase has a proofreading function which avoids mistakes during amplification. The *Taq* polymerase is known to create one mismatch in 1,000 base pairs.

Reaction conditions for the Phusion™ polymerase were the following:

template DNA	10 pg (plasmid) / 200 ng (genomic DNA)
forward primer	10 pmol
reverse primer	10 pmol
dNTPs (10 mM)	200 µM
Phusion™ polymerase	0.4 U
5 x reaction buffer	4 µl
H_2O_{deion}	ad 20 µl

Conditions using the *Taq* polymerase were used as followed:

template DNA	10 pg (plasmid) / 200 ng (genomic DNA)
forward primer	20 pmol
reverse primer	20 pmol
dNTPs (10 mM)	200 µM
Taq polymerase	1 U
10 x reaction buffer	2 µl
H_2O_{deion}	ad 20 µl

The lid of the PCR-machine was pre-headed to 10°C greater than the denaturation temperature. Before insertion of the reaction tubes, the PCR-machine was pre-headed to the denaturation temperature. After an initial DNA denaturation step, a cycle consisting of the three steps denaturation, primer annealing and primer elongation was completed 30 times. The reaction was terminated with a final elongation step. Time and temperature for denaturation, time of annealing and elongation temperature remained unchanged for each reaction. Annealing temperature (T_m) depended on oligonucleotide length and G+C content. It was calculated as follows:

$$T_m\ [°C] = 69.3 + 0.41\ (\%\ G+C) - 650/n$$

% G+C represents the G+C content in the primer; *n* represents the number of nucleotides.

To optimise the amplification yield for Phusion™ polymerase, the PCR reaction was performed in a gradient cycler.
The duration of the elongation step was chosen according to the length of the DNA fragment to be amplified. The *Taq* polymerase inserts approximately 1,000 nucleotides per minute, whilst the Phusion™ polymerase is faster with less than 30 s per 1,000 nucleotides.

Standard program for the Phusion™ polymerase:

denaturation	98°C	30 s	
denaturation	98°C	10 s	30x
annealing	T_m [°C] +/- 10°C	20 s	
elongation	72°C	30 s per 1 kb	
elongation	72°C	10 min	

Standard program for the *Taq* polymerase:

denaturation	94°C	60 s	
denaturation	94°C	30 s	30x
annealing	T_m [°C]	60 s	
elongation	72°C	60 s per 1 kb	
elongation	72°C	10 min	

II.5.6 Site-Directed Mutagenesis (QuikChange)

DNA bases on plasmids were exchanged using the method of site-directed mutagenesis. The QuikChange site-directed mutagenesis kit (Stratagene; La Jolla; USA) was employed in this study. The method utilises a dsDNA plasmid as template and two synthetic oligonucleotide primers. The primers contained the desired mutation and were complementary to opposite strands of the plasmid. During the PCR with *PfuTurbo* DNA polymerase (Stratagene; La Jolla; USA), a mutated plasmid was generated. The PCR product was treated with *Dpn*I, an endonuclease specifically digesting partially methylated DNA which was the template plasmid DNA in this case. This allowed isolation of the newly synthesised unmethylated DNA of the mutated plasmid which was subsequently transformed into a suitable *E. coli* strain. Here, *E. coli* DNA ligase closed the nicked DNA circles.

PCR reactions with the QuikChange site-directed mutagenesis kit were carried out according to the manufactorer's instructions using half of the reaction ingredients in half of the reaction volume. Mutated plasmids were transformed into super-competent *E. coli* XL1-Blue cells supplied with the kit.

Oligonucleotide primers were designed following the guidelines provided. They were supplied by biomers.net GmbH (Ulm; Germany). Primers are listed in **table 5**. Nucleotides exchanged in comparison with the original plasmid sequence are given in lower case.

Table 5: Oligonucleotide primers used for site-directed mutagenesis. Nucleotides exchanged in comparison with the original plasmid sequence are given in lower case.

Primer designation	Primer sequence (5' – 3')
pMM1522for	CAAAGGGGGAAATGtacAATGGTCCAAACTAGTTCG
pMM1522rev	CGAACTAGTTTGGACCATTgtaCATTTCCCCCTTTG
pSTOP1522for_I	GCTAACGGATTCACCggTCCAAGAATTGG

Table 5 (continued): Oligonucleotide primers used for site-directed mutagenesis. Nucleotides exchanged in comparison with the original plasmid sequence are given in lower case.

Primer designation	Primer sequence (5' – 3')
pSTOP1522rev_I	CCAAGCTTCTTGGAccGGTGAATCCGTTA
pSTOP1522for_II	CGCATGCCGGCTaaacCTCGCgAACGGATTCACCG
pSTOP1522rev_II	CGGTGAATCCGTTcGCGAGgtttAGCCGGCATGCG
p1533_Kas_for	CGTCTGGCGCAGGaGCCGCATTCGAAGATCTCC
p1533_Kas_rev	GGAGATCTTCGAATGCGGCtCCTGCGCCAGACG
LevΔ773_stop_for	CGATGCAAAACAAtaTCTGCAGCTGGTACC
LevΔ773_stop_rev	GGTACCAGCTGCAGATtaTTGTTTTGCATCG

II.5.7 Digestion of DNA with Restriction Endonucleases

Digestion of DNA was carried out using restriction endonucleases purchased from New England BioLabs (NEB; Ipswich; USA) or MBI Fermentas (MBI Fermentas; St. Leon-Rot; Germany). Reaction buffers, concentrations of enzymes and DNA as well as incubation temperatures were chosen according to the manufacturer's instructions. The digestion was allowed to proceed for up to 16 h and was, if possible, followed by heat inactivation of the restriction endonucleases (20 min; 65°C or 80°C).

II.5.8 Working with Oligonucleotides

For cloning DNA fragments smaller then 150 bp, oligonucleotides of up to 100 nucleotides were synthesised and purified by HPLC or PAGE (biomers.net GmbH; Ulm; Germany). For this purpose, both ssDNA strands of a dsDNA fragment were designed to form base overhangs at both ends of the fragment after their hybridisation. Corresponding ends were designed for insertion into a previously cut plasmid.

II.5.8.1 Phosphorylation of Oligonucleotides

Prior using the oligonucletides for cloning, the 5' end of each had to be phosphorylated. The 5' phosphate group is important for ligation into a previously cut and dephosphorylated plasmid.

For this purpose, 10 U of T4 polynucleotide kinase (New England BioLabs; Ipswich; USA), 5 µl of 10 x T4 DNA kinase buffer supplemented with 0.1 µM of ATP und 300 pmol of each oligonucleotide were filled up with H_2O_{deion} to a final volume of 50 µl. The phosphorylation reaction was incubated at 37°C for 30 min followed by a denaturation step at 65°C for 20 min.

II.5.8.2 Hybridisation of Oligonucleotides

For hybridisation of the corresponding oligonucleotides, the phosphorylation reactions of the pairwise matching oligonucleotides were mixed. After incubation at 95°C for 3 min, the annealing process took place at an appropriate hybridisation temperature (T_m) for 1 min. The calculation of T_m was performed as described in II.5.5. The hybridisation process was finished with a step at 55°C for 1 min and the hybridised oligonucleotides were used for ligation reaction (section II.5.10).
Oligonucleotides used in the study are listed in **table 6**. Nucleotides belonging to a certain recognition site for a restriction enzyme are *italicised*.

Table 6: Oligonucleotides used in this study. Nucleotides belonging to a certain recognition site for a restriction enzyme are *italicised*.

Oligonucleotide designation	Oligonucleotide sequence 5' – 3'
SP_lipA_forI	*GTACA*ATGAAAAAAGTACTTATGGCATTCAT
SP_lipA_revI	GTTTATTACTTACGGTATTCATGAAAAAAGTA*A*
SP_lipA_forII	TATTTGTTTATCGCTGATTCTATCTGTTTTAG
SP_lipA_revII	CGCCGATTTTGTCTATCTTAGTCGCTATTT
SP_lipA_forIII	CCGTCCGCCGTCCTGGCGCA*GGCGCC*GCA*TT*
SP_lipA_revIII	*GCTT*ACG*CCGCGG*ACGCGGTCTGCCGCCT
C-His_Term_for	*CGGCCG*CTCATCACCATCACCATCACTAAAAAGCCCTCAATGAAGAGGGC TTTTTTTA*A*
C-His_Term_rev	*CCGGT*TAAAAAAAGCCCTCTTCATTGAGGGCTTTTTAGTGATGGTGATGGT GATGAG*CGGCCGCATG*
C-Strep_Term_for	*CGGCCG*CTTGGAGCCACCCGCAGTTCGAGAAATAAAAAGCCCTCAATGAA GAGGGCTTTTTTTA*A*
C-Strep_Term_rev	*CCGGT*TAAAAAAAGCCCTCTTCATTGAGGGCTTTTTATTTCTCGAACTGCG GGTGGCTCCAAG*CGGCCGCATG*
N-His-TEV-rev	*C*CGG*AGATCT*G*GGCGCC*TTGGAAATATAAGTTTTCACCAGCTGAGTGATGG TGATGGTGATG*TTCGAAA*

Table 6 (continued): Oligonucleotides used in this study. Nucleotides belonging to a certain recognition site for a restriction enzyme are *italicised*.

Oligonucleotide designation	Oligonucleotide sequence 5' – 3'
N-His-TEV-for	*CTAGTTTCGAA*CATCACCATCACCATCACTCAGCTGGTGAAAACTTATATTTCCAA*GGCGCCCAGATCT*CCG*GAGCT*
N-Strep-TEV-for	*CTAGTTTCGAA*TGGTCACACCCACAATTCGAGAAATCAGCTGGTGAAAACTTATATTTCCAA*GGCGCCCAGATCT*CCG*GAGC*
N-Strep-TEV-rev	*C*CGG*AGATCT*G*GGCGCC*TTGGAAATATAAGTTTTCACCAGCTGATTTCTCGAATTGTGGGTGTGACCA*TTCGAAA*
C-Strep-for	*GCGGCCG*CTTGGAGCCACCCGCAGTTCGAGAAATAAAAAGCCCTCAATGAAGAGGGCTTTTTTTA*A*
C-Strep-rev	*CCGGT*TAAAAAAAGCCCTCTTCATTGAGGGCTTTTTATTTCTCGAACTGCGGGTGGCTCCAAGCGGCCG*CATGC*
N-Strep-Xa-for	CTAGTTTCGAATGGAGCCACCCGCAGTTTGAAAAAATCGAAGGGCGCCAGATCT
N-Strep-Xa-rev	CCGGAGATCTGGCGCCCTTCGATTTTTTCAAACTGCGGGTGGCTCCATTCGAAA
C-His-for	CCGGCCTGAGAGGATCGCATCACCATCACCATCACTAA
C-His-rev	CCGGTTTAGTGATGGTGATGGTGATGCGATCCTCTCAGGCCGGCATG
ASP_for	*GTACA*ATGCGTAAAGAAAAAAAGCATTAGCTGTAGCATTACTTTTAGCAGCATTAGGTGCATTACTTCCAACAACAGCATCTGCTGATACATTCGAAA*A*
ASP_rev	*GATCT*TTTCGAATGTATCAGCAGATGCTGTTGTTGGAAGTAATGCACCTAATGCTGCTAAAAGTAATGCTACAGCTAATGCTTTTTTTCTTTTACGCAT*T*

II.5.9 Purification of PCR Products and Plasmid Fragments

After PCR or plasmid digestion, an aliquot of the reaction mixture was analysed by agarose gel electrophoresis (section II.5.4). If only one DNA fragment was detected in the gel, the entire sample was subjected to purification with the QIAquick PCR purification kit (QIAgen; Hilden; Germany). If more than one DNA fragment was visible in the gel, the entire reaction mixture was separated gel electrophoretically. The DNA was visualised using the GelStar® Nucleic Acid Gel Stain (Biozym; Hessisch Oldendorf; Germany) on a blue light detector (Flu-O-blu) and a yellow filter instead of ethidium bromide. This avoids damaging the DNA by UV-light. The DNA fragment of interest was excised from the gel and purified using the QIAquick Gel Extraction Kit (QIAgen; Hilden; Germany).

All kits were used according to the manufacturer's instructions using H_2O_{deion} for elution of the DNA from the columns.

II.5.10 Ligation of DNA

To avoid re-circularisation of a previously digested DNA vector, the 5' phosphate groups of the linearised vector were removed prior to the ligation reaction. The dephosphorylation was achieved by adding 1 unit of calf intestinal alkaline phosphatase (CIP; New England BioLabs; Ipswich; USA) per µg of DNA to the sample immediately after restriction. An incubation at 37°C for 3 h followed. The DNA was purified using the PCR purification kit (QIAgen; Hilden; Germany) following the manufacturer's instructions.

In one ligation reaction, 25 – 200 ng of plasmid DNA were used. Insert DNA was added in excess (insert to vector ratio with regard to molar concentrations of 2 : 1 to 10 : 1) to a final volume of 8.5 µl. After incubation of the DNA for 5 min at 45°C, the reaction buffer supplied by the manufacturer and 200 U of T4 DNA ligase (New England BioLabs; Ipswich; USA) were added. The reaction was allowed to occur at 25°C for 20 min followed by 16°C for 16 h. After that, the ligation reaction was dialysed against H_2O_{deion} for 1 h at RT and was used for electroporation (section II.5.12.1) of competent *E. coli* cells.

II.5.11 DNA-Sequencing of Plasmid DNA

In all cases the successful modification of DNA – amplification followed by cloning as well as site-directed mutagenesis – was confirmed by sequence determination of the respective DNA region. The sequencing reactions were conducted by MWG Biotech AG or on site with an ABI PRISM 310 Genetic Analyser (Applied Biosystems; Perkin Elmer; Boston; USA). For latter, the required preparatory PCR with fluorescence-labelled ddNTPs and the purification of the PCR product were carried out as described by the manufacturer.

The analysis of all sequencing results was done using the software DNA Star (GATC Biotech; Konstanz; Germany).

All primers used for sequencing are listed in **table 7**.

Table 7: Oligonucleotide primers used for sequencing

Primer designation	Primer sequence (5' – 3')	Primer used for
SeqpMM1520_for	ATGATGAGATAAAGTTAGTTTATTGG	pMM1520 derivatives (cloning in MCS)
SeqpMM1520_rev	GTTTGCGCATTCACAGTTCTCC	pMM1520 derivatives (cloning in MCS)
Seq_prsA_rev	GGAATTCTTGAAGACGAAAGG	pRBBm20
Seq_AflII_for	GTGCTGTTTTATCCTTTACC	pMM1520 derivatives (cloning in *Afl*II)

Table 7 (continued): Oligonucleotide primers used for sequencing

Primer designation	Primer sequence (5' – 3')	Primer used for
Seq_AatII_rev	GAGCGGATACATATTTGAATG	pMM1520 derivatives (Cloning in *Aat*II)
Seq_pga1_for	GAATATGGGACAGAAGTTTCC	*pga* gene
Seq_pga2_rev	TGCTAATGAAGCCCCATAGC	*pga* gene
Seq_lev6_rev	TACAGGTACTGCATAGTAGG	*levΔ773* gene
Seq_lev5_for	TCTAAGTCTGCTGAAAAGGG	*levΔ773* gene
Seq_XhoI_for	CCGACTAGACCATAAGGG	pMM1520 derivatives (cloning in *Xho*I)
Seq_sacB_B.meg	CGTCTGTAACTGTGTTTG	$sacB_{B.meg}$ gene
Seq_pYZ5_for	GTGAAATACCGCACAGATGC	pMGBm19
Seq_pYZ5_rev	CATTGATTAATTTGCAGACAG	pMGBm19
Seq_pKM704_for	GAACGCCCGCAAGCCAACATG	pMGBm21

II.5.12 Transformation of Bacteria

II.5.12.1 Electroporation of *Escherichia coli* Cells

Starting out from an individual *E. coli* DH10B colony, a 5 ml overnight culture was inoculated. This culture was used to inoculate 500 ml of LB medium. The bacteria were incubated at 37°C and 200 rpm in baffled flasks until the culture reached an OD_{578nm} of 0.6. After cooling the cultures in ice water for 15 min, the cells were harvested by centrifugation (4,500 x g; 15 min; 4°C). The cells were washed twice with 20 ml of ice-cold water (2,600 x g; 8 min; 4°C) and suspended in 20 ml of 10 % (v/v) glycerol. After a further centrifugation step (2,600 x g; 8 min; 4°C), the obtained cells were dissolved in 1 ml of 10 % (w/v) glycerol. Competent cells were either used directly for transformation experiments or were shock frozen and stored at -80°C.

The competent *E. coli* cells were transformed with dialysed ligation reactions (section II.5.10) by electroporation with the help of a Gene Pulser™ apparatus (Bio-Rad; Munich; Germany). For this purpose, 20 – 200 ng of plasmid DNA were mixed with 40 µl of prepared competent *E. coli* cells in a 2 mm electroporation cuvette. The electroporation was carried out in the Gene Pulser™ at settings of 2.5 kV at 25 µF and 200 Ω. The transformed cells were regenerated for 1 h by incubation in 500 µl of LB medium at 37°C and smooth shaking. The

transformation volume was streaked out onto a LB medium agar plate with appropriate antibiotics. The plate was incubated overnight at 37°C.

II.5.12.2 Transformation of *Escherichia coli* Cells by the $CaCl_2$ Method

Starting out from an individual *E. coli* DH10B colony, a 5 ml overnight culture was inoculated. This culture was used to inoculate 100 ml of LB medium in a ration of 1 : 100. The bacteria were incubated at 37°C and 200 rpm in baffled flasks until the culture reached an OD_{578nm} of 0.8. After cooling the cultures on ice water for 10 min, the cells were harvested by centrifugation (2,600 x g; 10 min; 4°C). The cells were suspended in 10 ml of ice-cold 100 mM $CaCl_2$/10 % (w/v) glycerol and again cooled for 15 min in ice water. After centrifugation (2,600 x g; 10 min; 4°C), the cells were suspended in 1 ml of ice-cold 100 mM $CaCl_2$/10 % (w/v) glycerol. The competent cells were either used directly for transformation or were shock frozen and stored at -80°C.

The $CaCl_2$ prepared competent *E. coli* cells were transformed by heat shock. For this purpose, an aliquot of plasmid DNA was mixed with 50 µl of competent *E. coli* cells in a sterile reaction cup and was placed on ice for 20 min. After heating the cells for 45 s to 42°C, the cells were cooled down on ice for 2 min. To regenerate the transformed cells, they were incubated in 250 µl of LB medium at 37°C and smooth shaking for 1 h. The transformation volume was streaked out on a LB medium agar plate with appropriate antibiotics. The plate was incubated overnight at 37°C.

II.5.12.3 Protoplast Transformation of *Bacillus megaterium* Cells

Starting out from an individual *B. megaterium* colony, an overnight culture was inoculated (section II.4.4). An 1 ml aliquot of this culture was used to inoculate 50 ml of LB medium. The culture was incubated at 37°C and 250 rpm in a baffled flask until it reached an OD_{578nm} of 1.0. Cells were separated from the growth medium by centrifugation (2,600 x g; 15 min; 4°C) and suspended in 5 ml of freshly prepared SMMP. After adding 100 µl of freshly prepared sterile lysozyme solution (100 µg of lysozyme ml^{-1} in SMMP), the protoplast suspension was incubated at 37°C for 30 min and smooth shaking. Forming of protoplasts was controlled by microscope. After up to 80 % of the rod shaped bacterium cells have formed coccoid protoplasts, the protoplasts were harvested (1,300 x g; 10 min; RT). The supernatant was decanted carefully and the protoplasts were suspended in 5 ml of SMMP. After a second washing step, the protoplasts were suspended in 5 ml of SMMP and 750 µl of

87 % (w/v) glycerol were added. They were either used directly for transformation or were frozen and stored in portions of 500 µl at -80°C for a period of not longer than 2 months.
Before starting the transformation, protoplasts were tested for viability. Therefore, a 500 µl aliquot of protoplast solution was mixed with 2.5 ml of cR5-top agar as described below and was streaked out on a LB medium agar plate without antibiotics. After incubation overnight, a thick film of *B. megaterium* cells should be seen.
For transformation of the protoplasts, 5 µg of plasmid DNA were dissolved in 10 µl of SMMP for 20 min at 37°C. A 500 µl aliquot of protoplasts suspension was mixed with the DNA and was given into 1.5 ml of PEG-P solution. After incubation for 2 min at RT, 5 ml of SMMP were added and the reaction was mixed gently. The protoplasts were harvested by centrifugation (1,300 x g; 10 min; RT), carefully suspended in 500 µl of SMMP and incubated at 30°C for 45 min without shaking followed by 45 min of smooth shaking at 300 rpm (Thermomixer compact; Eppendorf; Germany). The regenerated protoplasts were mixed with 2.5 ml of 42°C cR5-top agar and given on a pre-headed LB medium agar plate containing the required antibiotics. For outgrowth, the plates were incubated at 30°C for up to 24 h. Colonies seen after this period of incubation were streaked out on new LB medium agar plates containing the required antibiotics.

II.5.12.3.1 Solutions for Protoplast Transformation

SMMP	2 x AB3 and 2 x SMM; mixed 1 : 1	
2 x AB3	Antibiotic medium No. 3 (Difco)	35.0 g l^{-1}
2 x SMM (pH 6.5)	Malic acid	40.0 mM
	$MgCl_2$ x 6 H_2O	40.0 mM
	NaOH	80.0 mM
	Sucrose	1.0 M
	dissolved in H_2O_{deion}, sterilised by filtration	
PEG-P solution	PEG 6000	40.0 % (w/v)
	dissolved in 1 x SMM (pH 6.5)	
cR5 top-agar (2.5 ml)	Solution A	1.25 ml
	Solution B	713.0 µl
.	8 x cR5-salts	288.0 µl
	L-proline (12 % (w/v))	125.0 µl
	D-glucose (20 % (w/v))	125.0 µl

Solution A (pH 7.3)	Sucrose	602.0 mM
	MOPS	58.0 mM
	NaOH	30.0 mM
	dissolved in H_2O_{deion}, sterilised by filtration	
Solution B	Agar agar	4.0 % (w/v)
	Casamino acids	0.2 % (v/w)
	Yeast extract	10.0 % (v/w)
	dissolved in H_2O_{deion}	
8 x cR5-salts	K_2SO_4	11.0 mM
	$MgCl_2$ x 6 H_2O	394.0 mM
	KH_2PO_4	3.0 mM
	$CaCl_2$	159.0 mM
	dissolved in H_2O_{deion}	

II.5.13 Plasmid Construction

II.5.13.1 Plasmids for Intracellular Production of Recombinant Proteins

Primers pMM1522for and pMM1522rev (**Tab. 5**) were used for site-directed mutagenesis (section II.5.6) of *B. megaterium* expression plasmid pMM1520 (Malten *et al.*, 2005a) to generate a new restriction site for *Bsr*GI directly upstream of the start codon of the xylose-inducible open reading frame. The new vector was named **pMM1522**. For the construction of **pSTOP1522**, an *Age*I restriction site and a stop codon with a 3 bp downstream *Nru*I restriction site were successively introduced into **pMM1522** by site-directed mutagenesis. Here, the primer pairs pSTOP1522for_I/rev_I and pSTOP1522for_II/rev_II, respectively (**Tab. 5**) were applied.

The DNA sequences of certain affinity tags were introduced into the expression vector **pSTOP1522** using the synthetic oligonucleotide strategy as described in II.5.8. For the construction of expression vector **pSTREP1522**, oligonucleotides N-Strep-Xa-for and N-Strep-Xa-rev (**Tab. 6**) encoding the *Bst*BI restriction site, the StrepII-tag, the Factor Xa protease cleavage site and the *Kas*I and the *Bgl*II restriction site were hybridised and inserted into the *Spe*I/*Sac*I digested vector **pSTOP1522**. Expression vector **pHIS1522** was obtained by insertion of the hybridised oligonucleotides C-His-for and C-His-rev (**Tab. 6**) encoding a linker region followed by the His_6-tag and a stop codon into *Sph*I/*Age*I digested pMM1522.

For construction of the expression plasmid **pSTOP1622**, an 855 bp long *Afl*II fragment was eliminated from the *B. megaterium* expression plasmid **pSTOP1522** and the remaining 6,535 bp vector was religated. The new plasmid was called **pSTOP1622**. Using the same strategy, plasmids **pHIS1522** and **pSTREP1522** were shortened resulting in **pC-HIS1622** and **pN-STREP-Xa1622**, respectively. Synthetic oligonucleotides encoding further affinity tags were annealed and introduced into the expression vector **pSTOP1622** as described in II.5.8. For the construction of expression vector **pC-STREP1622**, oligonucleotides C-Strep-for and C-Strep-rev (**Tab. 6**) encoding a *Not*I site followed by the StrepII-tag were hybridised and inserted into the *Sph*I and *Age*I digested vector **pSTOP1622**. The hybridised oligonucleotides N-Strep-TEV-for and N-Strep-TEV-rev (**Tab. 6**) encoding the *Bst*BI restriction site, the StrepII-tag, the TEV-protease cleavage site and the *Kas*I and the *Bgl*II restriction sites were inserted into the *Spe*I/*Sac*I digested **pSTOP1622** resulting in expression vector **pN-STREP-TEV1622**. For construction of expression vector **pN-HIS-TEV1622**, oligonucleotides N-His-TEV-for and N-His-TEV-rev (**Tab. 6**) encoding the *Bst*BI site, the His_6-tag, the TEV-protease cleavage site and the *Kas*I and the *Bgl*II restriction site were hybridised and inserted into the *Spe*I/*Sac*I digested vector **pSTOP1622** (**Fig. 8A**).

For **pMGBm19**, *xylR*, the promoter P_{xylA} and the MCS of **pMM1520** were amplified using the primers XylR_for_YZ5 and MCS_rev_YZ5 (**Tab. 4**) including the *Nar*I and the *Bam*HI restriction site, respectively. After digestion and purification, the 1,450 bp fragment was inserted into the *Bam*HI/*Nar*I digested **pYZ5**. Furthermore, a 1,555 bp fragment containing *xylR*, P_{xylA} and the MCS of **pMM1520** was applied using primers XylR_for_KM704 and MCS_rev_KM704 (**Tab. 4**) including the *Eco*RV and the *Pst*I restriction site, respectively. After digestion and purification, the product was inserted into the *Eco*RV/*Pst*I digested **pKM704** resulting in **pMGBm21** (**Fig. 8B+C**).

II.5.13.1.1 Plasmid Construction for the Production of the Green Fluorescent Protein (GFP) in *Bacillus megaterium*

The gene of the green fluorescent protein (GFP) *gfp* was introduced into the various constructed vectors described in section II.5.13.1.

For **pRBBm34**, *gfp* including its original stop codon, was amplified by PCR using primers GFP+for and GFP+rev (**Tab. 4**) with pMUTIN-GFP+ (Bacillus Genetic Stock Center; Columbus; Ohio; USA) as template. Primer GFP+for included the *Bgl*II site at its 5' end followed by two guanidine residues important for cloning the target gene in the correct

reading frame, whilst primer GFP+rev included the *Eag*I site at its 5' end. After digestion and purification, the PCR product was inserted into the *Bgl*II/*Eag*I digested **pMM1522**.

Furthermore, *gfp* was amplified by PCR using primers GFP+for and GFP+revII (**Tab. 4**) with pMUTIN-GFP+ as template. The *Sph*I site was encoded in the 5' end of primer GFP+revII followed by two adenosine residues important for cloning of the target gene into the correct reading frame in accordance to the following coding sequence. Using primer GFP+revII, the amplified *gfp* lacked its original stop codon which made it usable for fusions to 3' coding sequences. Cloning of the *Bgl*II/*Sph*I digested and purified PCR fragment into the appropriately *Bgl*II/*Sph*I cut **pSTOP1622**, **pC-HIS1622**, **pN-STREP-Xa1622**, **pN-HIS-TEV1622**, **pN-STREP-TEV1622**, and **PC-STREP1622** resulted in **pRBBm53**, **pRBBm35**, **pRBBm36**, **pRBBm54**, **pRBBm55**, and **pRBBm56**, respectively.

For **pRBBm63** and **pRBBm64**, *gfp* was amplified by PCR with pMUTIN-GFP+ as template using primer pairs GFP_for_SpeI/GFP_rev_XmaI (**Tab. 4**) including the *Spe*I and the *Xma*I restriction site, respectively, and GFP_for_XmaI/GFP_rev_EagI (**Tab. 4**) including the *Xma*I and the *Eag*I restriction site, respectively (**Fig. 8A**). After digestion and purification, the PCR products were inserted into the appropriately *Spe*I/*Xma*I cut **pMGBm19** and *Xma*I/*Eag*I cut **pMGBm21** resulting in **pRBBm63** and **pRBBm64**, respectively (**Fig. 8B+C**).

Using genomic DNA of *B. megaterium* DSM319 as template, the coding sequence of thioredoxin $trxA_{B.meg}$ was amplified by PCR using primers Thio-GFP_for and Thio-GFP_rev (**Tab. 4**). Both primers included the *Spe*I restriction site at their 5' ends. Cloning of the *Spe*I digested PCR-fragment into the previously cut **pRBBm54** and **pRBBm55** resulted in **pRBBm67** and **pRBBm68**, respectively. To approve the right orientation of the inserted PCR fragment in frame to the previously inserted coding sequences, new achieved plasmids were checked by a *Xho*I/*Pac*I double digestion. *Pac*I cut next to the 3' end of $trxA_{B.meg}$, while *Xho*I was a single restriction site in **pRBBm67** and **pRBBm68**. Based on the electrophoretical analysis of the digestion products, 50 % of the checked clones showed the right orientation of $trx_{B.meg}$ (**Fig. 8A**).

For **pRBBm40**, *repU* and *oriU* of **pMM1520** were amplified using primer pairs RepU1520_for and RepU1520_rev (**Tab. 4**). Both primers included the *Afl*II restriction site at their 5' ends. Cloning of the *Afl*II digested and purified PCR-fragment into the *Afl*II cut pMUTIN-GFP+ resulted in **pRBBm40**. Orientation of the inserted PCR product did not matter (**Fig. 8D**).

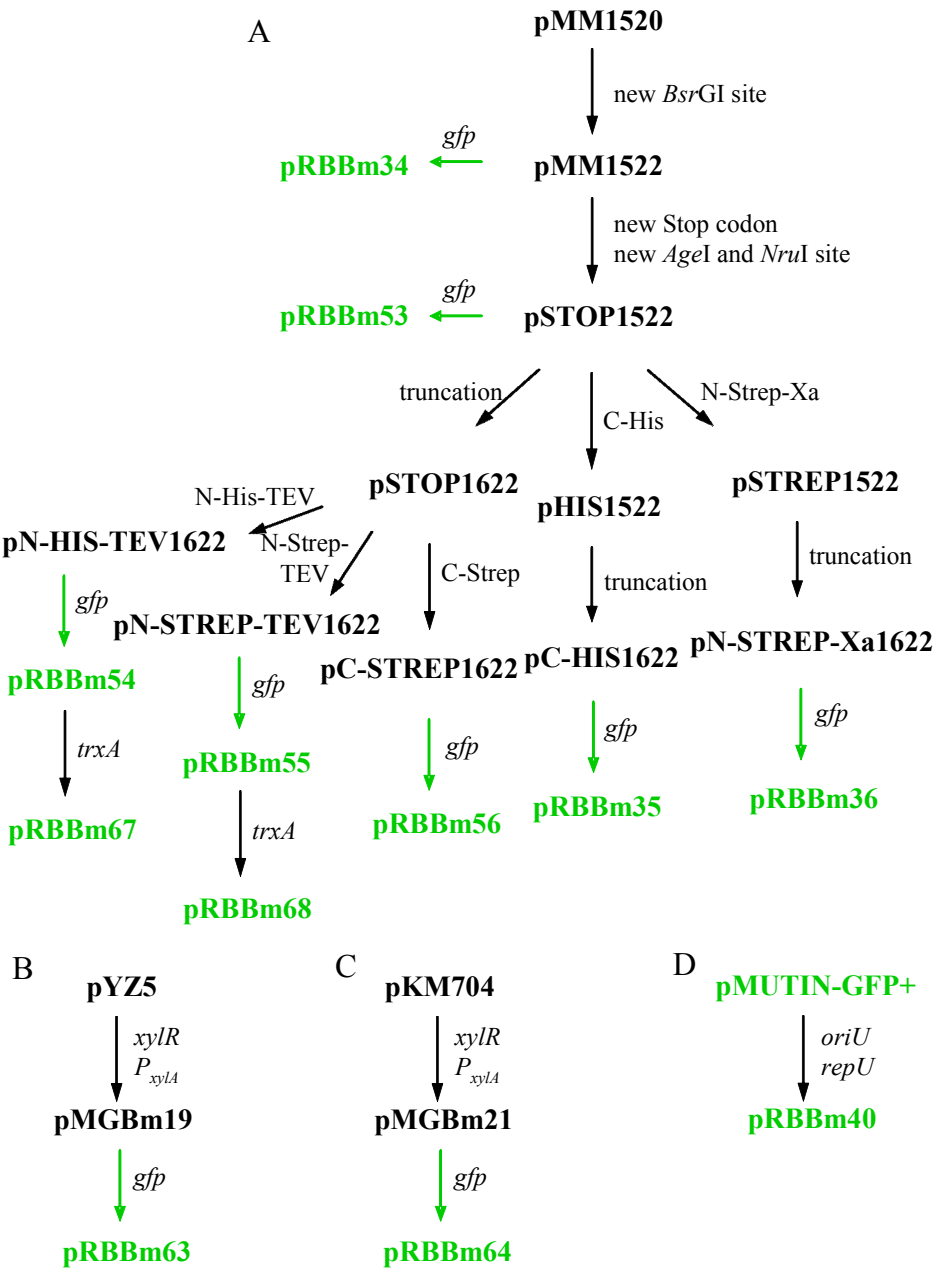


Figure 8: Construction scheme of vectors for the intracellular GFP production in *B. megaterium*. For further details consult **table 2** and sections II.5.13.1 and II.5.13.1.1. Arrows are indicating cloning direction. Gene names next to the arrows indicate inserted fragments. Green: *gfp* containing plasmids.

II.5.13.1.2 Plasmids for Promoter Deletion Analysis of $P_{sacBB.meg}$

For analysis of *B. megaterium* DSM319 promoter $P_{sacBB.meg}$, **pRBBm34** was used as promoter test vector. For **pRBBm59**, **pRBBm70**, **pRBBm71**, and **pRBBm72**, the coding sequence of the region containing $P_{sacBB.meg}$ including the DNA for the first 10 N-terminal amino acids of *sacB* from *B. megaterium* DSM319 were amplified using PsacB_rev in combination with

PsacB_for, PsacB_forI, PsacB_forII, or PsacB_forIII (**Tab. 4**) with genomic DNA of *B. megaterium* DSM319 as template. PsacB_rev included the *Spe*I site restriction site at its 5' end and PsacB_for, PsacB_forI, PsacB_forII, and PsacB_forIII the *Afl*II site. After appropriate digestion and purification of the PCR products, the resulting fragments of 676 bp, 316 bp, 221 bp, and 122 bp were cloned into the *Spe*I/*Afl*II 6,646 bp of **pRBBm34** resulting in **pRBBm59**, **pRBBm70**, **pRBBm71**, and **pRBBm72**, respectively (**Fig. 9**).

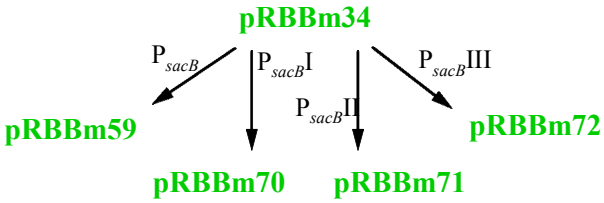


Figure 9: Construction scheme of vectors for the promoter deletion analysis of $P_{sacBB.meg}$ in *B. megaterium*. For further details consult **table 2** and section II.5.13.1.2. Arrows are indicating cloning direction. Gene names next to the arrows indicate inserted fragments. Green: *gfp* containing plasmids

II.5.13.2 Plasmids for Extracellular Production of Recombinant Proteins

The secretion vector **pMM1525** coding for the signal peptide (SP_{lipA}) of the *B. megaterium* extracellular esterase LipA was created by insertion of three pairs of hybridised synthetic oligonucleotides (section II.5.8) (oligonucleotide pairs SP_lipA_forI/revI, SP_lipA_forII/revII and SP_lipA_forIII/revIII). The double stranded oligonucleotides were ligated to each other resulting in an 84 bp long ds oligonucleotide coding for SP_{lipA}. This was ligated into the *Bsr*GI/*Bst*BI digested vector **pMM1522**. The resulting vector encoding for SP_{lipA} but missing the *Bst*BI restriction site was named **pMM1525**.

During the construction of **pMM1525**, the mutant plasmid **pMM1533** with the additional *Bst*BI site downstream the DNA sequence encoding SP_{lipA} but without the *Bsr*GI site was isolated. In order to prepare the vector for the in frame insertion of target genes downstream of the affinity tag DNA sequence, the *Sfo*I site of **pMM1533** was eliminated by a site-directed mutagenesis using the primers p1533_Kas_for and p1533_Kas_rev (**Tab. 5**) resulting in **pADBm5**. The 6,861 bp and the 640 bp DNA fragments obtained by the *Xho*I/*Bst*BI digest of the plasmids **pSTREP1522** (StrepII-tag) (section II.5.13.1) and **pADBm5** (signal peptide SP_{lipA}), respectively, were combined and ligated in a way that they maintained the unique *Sfo*I site downstream of the StrepII-tag coding sequence. The resulting plasmid was named **pSTREP1525**. For the construction of **pHIS1525**, 643 bp and 6,831 bp fragments from the

*Xho*I/*Bgl*II digested **pMM1525** and **pHIS1522** (section II.5.13.1) were combined and ligated. The affinity tags of **pHIS1525** and **pSTREP1525** were combined using the outlined strategy resulting in **pSTREPHIS1525** (**Fig. 10**).

II.5.13.2.1 Insertion of the Coding Sequence of the Levansucrase LevΔ773 from *Lactobacillus reuteri* into *Bacillus megaterium* Expression Vectors

Next, the part of the *L. reuteri* levansucrase gene encoding an active and soluble protein in *E. coli* was introduced into the various constructed vectors. The gene *levΔ773*MycHis including the Myc-epitope and the His_6-tag coding sequence was amplified by PCR using primers levΔ773_for and levΔ773_rev (**Tab. 4**) with **pBAD-*lev*** as template (van Hijum *et al.*, 2001). Primer levΔ773_for included the *Sac*I followed by the *Sfo*I restriction site at its 5' end, whilst the *Sph*I site was encoded in primer levΔ773_rev. Cloning of the *Sac*I/*Sph*I digested PCR fragment into the appropriately cut **pMM1525** resulted in **pMMBm6**. This plasmid still contained a DNA linker between the coding sequences of the signal peptide and the target gene *levΔ773*MycHis. Since this linker contained two *Sfo*I sites, it was eliminated by a *Sfo*I digestion and religation. The resulting plasmid was named **pMMBm7** and encoded SP_{lipA} directly upstream of *levΔ773*MycHis. A plasmid encoding LevΔ773 without Myc- and His_6-tag was constructed by insertion of a stop codon directly downstream of *levΔ773* by site-directed mutagenesis (section II.5.6) using primers LevΔ773_stop_for and LevΔ773_stop_rev (**Tab. 5**). The resulting plasmid **pMGBm4** was identified due to the concomitant elimination of the *Bgl*II restriction site (**Fig. 10**).

The *levΔ773* gene was further amplified by PCR using primers levΔ773_forI and levΔ773_revI (**Tab. 4**) with **pMGBm4** as template. Primer levΔ773_forI included a *Bgl*II restriction site at its 5' end followed by two cytosine residues important for cloning of the target gene in the correct reading frame. The *Sph*I restriction site was inserted into the 5' end of primer levΔ773_revI. Cloning of the *Bgl*II/*Sph*I digested PCR fragment into the *Bgl*II/*Sph*I restricted **pSTREP1525** and **pSTREPHIS1525** resulted in **pRBBm13** and **pRBBm16**, respectively. For **pRBBm15** *levΔ773* was amplified by PCR using primers levΔ773_for and levΔ773_revI (**Tab. 4**). Here, the same strategy was employed as for the construction of **pMMBm7** from **pMMBm6**. Cloning of the *Sac*I/*Sph*I digested PCR fragment into the *Sac*I/*Sph*I cut **pHIS1525** resulted in **pRBBm14**. The linker region between the coding sequences of the signal peptide and *levΔ773* of **pRBBm14** was eliminated as described for **pMMBm7** above to result in **pRBBm15** (**Fig. 10**).

For the integration of a terminator sequence, oligonucleotides C-His_Term_for and C-His_Term_rev (**Tab. 6**) were used to insert DNA encoding a *Nco*I site, a C-terminal His_6-tag and a terminator into *Sph*I/*Age*I restricted **pRBBm15**, resulting in **pEJBm7**. Furthermore, oligonucleotides C-Strep_Term_for and C-Strep_Term_rev (**Tab. 6**) were used to insert DNA encoding a *Nco*I site, a C-terminal StrepII-tag and a terminator into the *Sph*I/*Age*I restricted **pRBBm15** replacing the His_6-tag coding sequence, resulting in **pEJBm2** (**Fig. 10**).

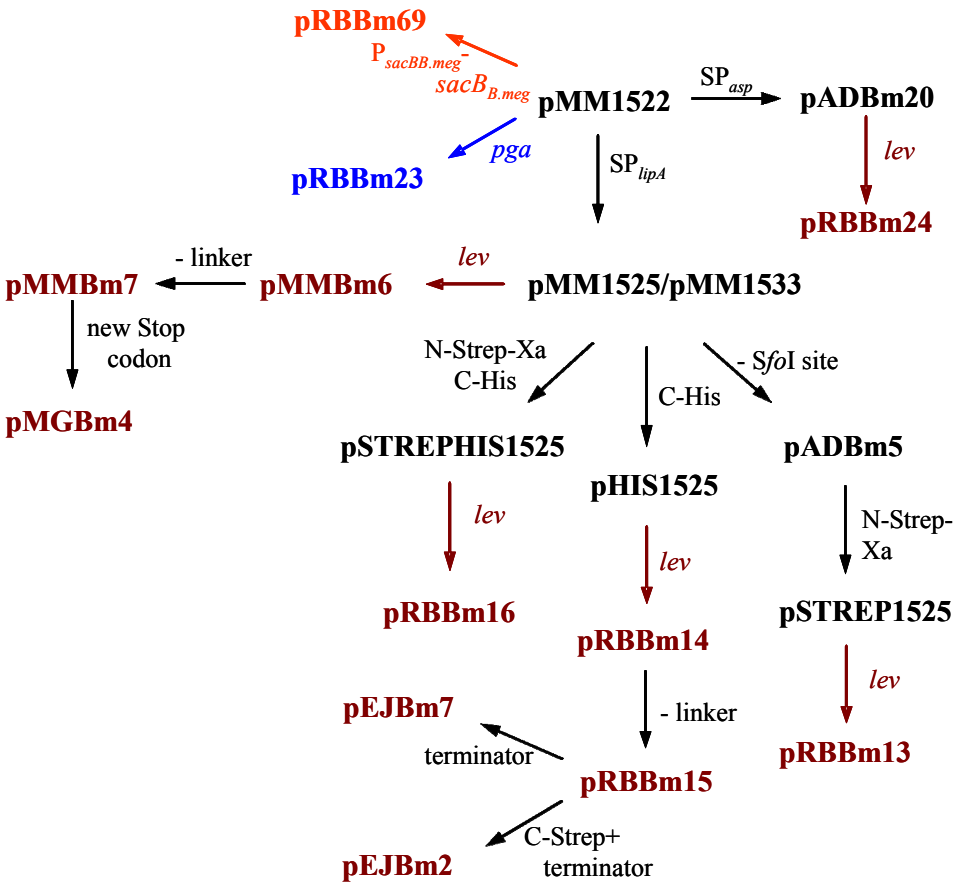


Figure 10: Construction scheme of secretion vectors for the extracellular production of recombinant proteins in *B. megaterium*. For further details consult **table 2** and section II.5.13.2. Arrows are indicating cloning direction. Gene names next to the arrows indicate inserted fragments. Brown: *lev*Δ*773* containing plasmids. Blue: *pga* containing plasmid. Red: $sacB_{B.meg}$ containing plasmid.

II.5.13.2.2 Insertion of the Coding Sequence of the Penicillin G Acylase PGA of *Bacillus megaterium* ATCC14945 into *Bacillus megaterium* Expression Vectors

Next, the gene of the penicillin G acylase PGA *pga* including the sequence of its natural signal peptide SP_{pga} of *B. megaterium* strain ATCC14945 (Panbangred *et al.*, 2000) was

cloned to create **pRBBm23** (**Fig. 10**). The corresponding DNA *sp$_{pga}$-pga* was amplified using Oligo_pga_for and Oligo_pga_rev (**Tab. 4**) with genomic DNA of *B. megaterium* ATCC14945 as template. Oligo_pga_for included the *Bsr*GI and Oligo_pga_rev the *Sac*I restriction site at their 5' ends. Cloning of the *Bsr*GI/*Sac*I digested PCR fragment into the appropriately cut **pMM1522** resulted in **pRBBm23**. For new secretion vectors, the coding sequence of the signal peptide SP$_{pga}$ and parts of different lengths of the PGA α-subunit were amplified using primer Sp_pga_for in combination with the primers Sp_pga_rev, Sp_pgaI_rev, Sp_pgaII_rev, and Sp_pgaIII_rev (**Tab. 4**), respectively, with **pRBBm23** as template. Primers Sp_pga_rev, Sp_pgaI_rev, Sp_pgaII_rev, and Sp_pgaIII_rev included the *Spe*I restriction site at their 5' ends. The PCR products were digested using the restriction enzymes *Xho*I and *Spe*I resulting in fragments of 633 bp (*sp$_{pga}$*), 840 bp (*sp$_{pga}$-α-subunit''*), 1,179 bp (*sp$_{pga}$-α-subunit'*), and 1,348 bp (*sp$_{pga}$-α-subunit*), respectively. After purification, the fragments were cloned into the appropriately cut **pSTREP1522** resulting in **pRBBm26**, **pRBBm27**, **pRBBm28**, and **pRBBm29**, respectively. To fuse the signal peptide derivatives fragments to *levΔ773*, the *Bgl*II/*Sph*I-fragment (2,222 bp) of **pRBBm13** was subcloned into **pRBBm26**, **pRBBm27**, **pRBBm28**, and **pRBBm29** resulting in **pRBBm30**, **pRBBm31**, **pRBBm32**, and **pRBBm33**, respectively (**Fig. 11**).

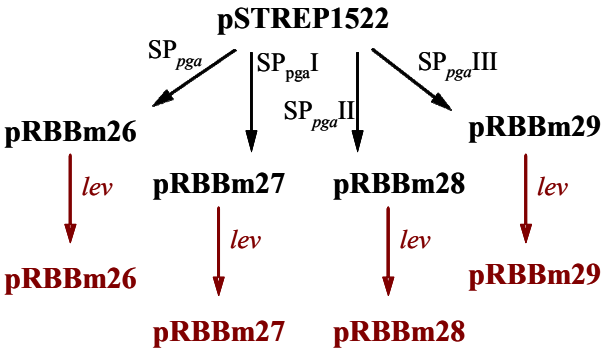


Figure 11: Construction scheme of secretion vectors for the extracellular production of LevΔ773 directed by the SP$_{pga}$ in *B. megaterium*. For cloning further details consult **table 2** and section II.5.13.2.2. Arrows are indicating cloning direction. Gene names next to the arrows indicate inserted fragments. Brown: *levΔ773* containing plasmids.

II.5.13.2.3 Constructions of Further Secretion Vectors

Plasmid **pADBm20**, a **pMM1522** derivative containing the DNA sequence coding for an artificial signal peptide (SP$_{asp}$) was constructed using the oligonucleotides ASP_for and

ASP_rev (**Tab. 6**). After hybridisation of the oligonucleotides, the dsDNA fragment was cloned into the *Bsr*GI/*Sph*I cut **pMM1522** resulting into **pADBm20** (**Fig. 10**). The *Xho*I/*Bgl*II fragment of **pADBm20** containing sp_{asp} was cloned into the previously cut *Xho*I/*Bgl*II **pRBBm13** lacking sp_{lipA}. The new vector coding SP_{asp}-StrepII-Xa-*levΔ773* was named **pRBBm24**.

For insertion of the gene of the signal peptidase SipM of *B. megaterium sipM* under the control of its own promoter, a 6,569 bp *Sph*I/*Xho*I *sipM* gene containing fragment was subcloned from **pMMBm3** (Malten *et al.*, 2005b) to the 2,999 bp *Xho*I/*Sph*I fragment of **pMMBm7** and to the 2,850 bp *Xho*I/*Sph*I fragment of **pEJBm2** resulting in **pRBBm19** and **pRBBm46**, respectively. Furthermore, a 4,649 bp *Aat*II/*Xho*I fragment was subcloned from **pMMBm3** to the 4,965 bp *Xho*I/*Aat*II fragment of **pRBBm23** resulting in **pRBBm17** (**Fig. 12**).

For insertion of the gene coding for the chaperon PrsA of *B. subtilis* under control of its own promoter, a 7,069 bp *Sph*I/*Xho*I *prsA* gene containing fragment was subcloned from **pMMBm9** (Malten, 2005) to the 2,999 bp *Xho*I/*Sph*I fragment of **pMMBm7** resulting in **pRBBm20**. For **pRBBm18**, the 7,049 bp *Sac*I/*Xho*I fragment was subcloned from **pMMBm9** to the 3,022 bp *Xho*I/*Sac*I fragment of **pRBBm23** (**Fig. 12**).

Figure 12: Construction scheme of secretion vectors for the extracellular production of recombinant proteins with coproduction of SipM or PrsA in *B. megaterium*. For cloning further details consult **table 2** and section II.5.13.2.3. Arrows are indicating cloning direction. Gene names next to the arrows indicate inserted fragments. Brown: *levΔ773* containing plasmid. Blue: *pga* containing plasmid. Violet: *sipM* containing plasmids. Turquoise: *prsA* containing plasmids.

For **pRBBm69,** primer pair PsacB_for/pRBEc1_rev (**Tab. 4**) and genomic DNA of *B. megaterium* DSM319 as template were used to amplify a 2,107 bp fragment containing the promoter region of $sacB_{B.meg}$ and the coding sequence of SacB from *B. megaterium* $sacB_{B.meg}$. Primer PsacB_for included the *Afl*II site at its 5' end, whilst the *Bam*HI site was inserted into the 5' end of pRBEc1_rev. The *Afl*II/*Bam*HI digested and purified PCR fragment was inserted into the 5,059 bp *Bam*HI/*Afl*II fragment of **pMM1522** resulting in **pRBBm69** (**Fig. 10**).

II.6 RECOMBINANT PROTEIN PRODUCTION

II.6.1 Recombinant Protein Production in *Bacillus megaterium* Using the Xylose or Sucrose Inducible Promoter System

For all expression experiments, *B. megaterium* plasmid strains were streaked onto LB medium agar plates containing the required antibiotics (section II.4.2). Plates were incubated for 16 h at 37°C. Negative controls in all expression experiments were the *B. megaterium* strains MS941, WH320 or WH323 carrying the target gene less plasmids pMM1522, pMM1622 or pMGBm19. A liquid pre-culture was inoculated from a single *B. megaterium* colony and incubated at 37°C (section II.4.4).

For intracellular protein production, 50 ml of LB medium were supplemented with the required antibiotics. An 1 ml aliquot of the pre-culture was used to inoculate the main culture. For extracellular protein production, 100 ml or 500 ml of growth medium were supplemented with the required antibiotics and inoculated in the ratio of 1 : 100 with the pre-culture.

The cultures were incubated at 30°C or 37°C at 250 rpm. After reaching an OD_{578nm} of 0.4, recombinant gene expression was induced by adding 0.5 – 1.0 % (w/v) xylose or 0.5 % (w/v) sucrose to the growth medium. For measurement of the OD_{578nm} and for analysis of the intra- and extracellular protein fractions, samples were taken at different time points after induction of recombinant gene expression. For intracellular protein production, the incubation occurred in a water bath shaker (Aquatron; Infors AG; Bottmingen; Switzerland). Incubation for extracellular protein production took place on a bench top shaker (TR; Infors AG; Bottmingen; Switzerland).

II.6.1.1 Analysis of the Intracellular Protein Fraction

For analysis of the intracellular protein fractions, samples containing 3 x 10^9 *B. megaterium* cells were taken. After harvesting the cells (14,000 x g; 15 min; 4°C), the supernatant was completely removed. The cells were suspended in 30 µl of lysis buffer supplemented 2 µl ml^{-1} benzonase (Merck; Darmstadt; Germany) and incubated at 37°C for 30 min and 1,000 rpm (Thermomixer compact; Eppendorf; Germany). The cell disruption was enhanced by periodic vigorous shaking. After centrifugation (14,000 x g; 30 min; 4°C), 26 µl of the supernatant containing the soluble proteins were mixed with 13 µl of SDS loading dye and stored at RT. The sediment, containing cell debris and insoluble proteins, was suspended in 30 µl of urea-buffer and centrifuged for eliminating cell debris (14,000 x g; 30 min; 4°C). For analysis of the insoluble protein fraction, 26 µl of the supernatant were mixed with 13 µl of SDS loading

dye. 7.5 µl of the prepared samples which corresponded to proteins of 0.5 x 10^9 cells were analysed by SDS-PAGE (section II.6.3).

II.6.1.2 Analysis of the Extracellular Protein Fraction

For analysis of the extracellular proteins produced by *B. megaterium*, samples of 5 ml cell culture were taken at indicated time points after induction of gene expression. Cells were separated from the growth medium by centrifugation (2,600 x g; 15 min; 4°C). Proteins of the cell-free growth medium were precipitated by different methods.

The proteins from the cell-free growth medium were precipitated with acetone. Therefore, 3 ml of the cell-free growth medium were combined with 12 ml ice-cold acetone and stored at -20°C overnight. Precipitated proteins were collected by centrifugation (2,600 x g; 10 min; 4°C), dried at 37°C for 10 min and washed with 500 µl of H_2O. After drying, the proteins were suspended in 10 µl of urea-buffer and 10 µl of SDS loading dye. An aliquot of 10 µl of this sample containing the proteins of 1.5 ml of cell-free growth medium was analysed by SDS-PAGE (section II.6.3).

A second method for protein concentration from cell-free growth medium was lyophilisation. 2.5 ml of cell-free growth medium were desalted using a PD-10 column (GE Healthcare; Uppsala; Sweden) and subsequently lyophilised. The dried proteins were solubilised in 10 µl of urea-buffer and 15 µl of SDS loading dye. 15 µl containing the proteins of 1.5 ml cell-free growth medium were used for SDS-PAGE analysis (section II.6.3).

Using a third method, 660 mg (60 % (w/v)) extremely fine ammonium sulphate powder were added to 1.5 ml of cell-free LB or TB growth medium (Englard and Seifter, 1990). For precipitation of proteins from 1.5 ml of cell-free A5 or A5-MOPSO growth medium, these media were supplemented with tryptone to a final concentration of 10 g l^{-1} to enhance the polypetide concentration before adding the ammonium sulphate powder. A gently mixing was followed by an incubation for 2 h at 4°C and 1,000 rpm (Thermomixer compact; Eppendorf; Germany). Precipitated proteins were collected by centrifugation (14,000 x g; 30 min; 4°C). After removal of the supernatant, the proteins were suspended in 10 µl of urea-buffer and 5 µl of SDS loading dye and analysed by SDS-PAGE (section II.6.3). For recombinant protein purification from the cell-free LB growth medium, the volume was scaled up to 30 ml. After protein precipitation and harvesting, proteins were dissolved in a suitable buffer for purification process (section II.6.7.2).

For analysis of the secretome by two dimensional (2D) gel electrophoresis (section II.6.4), a trichloroacetic acid (TCA) precipitation followed the ammonium sulphate precipitation

avoiding high salt concentrations. The salts disturbed the 2D gel electrophoresis. The proteins of 9 ml of cell-free growth medium were precipitated by ammonium sulphate. The dried proteins were dissolved in 30 µl of H_2O_{deion}. 3 µl of 100 % TCA were added, gently mixed and stored at 4°C overnight. The precipitated proteins were collected by centrifugation (14,000 x g; 10 min; 4°C) and washed three times with acetone supplemented with bromophenol blue. The colour indicated the complete removal of the acid because low pH was shown by colour changing from blue to yellow. The proteins were dried and used for 2D gel electrophoresis (see II.6.4). Proteins of 25 times concentrated growth medium (section II.6.2) were also precipitated with a final concentration of 10 % (w/v) TCA. The dried proteins were dissolved in urea-buffer and SDS loading dye for SDS-PAGE analysis.

II.6.1.3 Solutions for Protein Analysis

Lysis buffer (pH 6.5)	Na_3PO_4	100.0 mM
	EDTA	20.0 mM
	Lysozyme	5.0 mg ml^{-1}
	Benzonase (25 U μl^{-1})	2.0 µl ml^{-1}
	dissoved in H_2O_{deion}	
SDS loading dye	Tris-HCl	250.0 mM
	SDS	8.0 % (w/v)
	Glycerol	25.0 % (w/v)
	β-mercaptoethanol (where indicated)	8.0 % (v/v)
	Bromophenol blue	350.0 µM
	dissolved in H_2O_{deion}	
Urea-buffer	Tris-HCl (pH 7.5)	50.0 mM
	Urea	8.0 M
	dissolved in H_2O_{deion}	

II.6.2 Concentration of Secreted Proteins by Cross-Flow Filtration of the Cell-Free Growth Medium

For concentration of secreted proteins, 2 litres of cell culture were separated in cells and growth medium by centrifugation (4,500 x g; 15 min; 4°C). The growth medium was filtrated by cross-flow filtration (GE Healthcare; Uppsala; Sweden) using a 0.2 µm filter unit to remove the remaining cells and cell debris. Using a membrane unit with a cut off value of 50

kDa, proteins of the cell-free growth medium with a relative molecular mass above 50,000 were concentrated 25 times. Diafiltration of the concentrate against buffer was performed under the same conditions with the used buffer containing 100 mM sodium phosphate and 50, 150, and 300 mM NaCl, respectively. After TCA precipitation (section II.6.1.2), proteins of 60 µl concentrated growth medium, equal to 1.5 ml not concentrated growth medium, were analysed by SDS-PAGE (section II.6.3). For determination of the total amount of secreted proteins in the cell-free growth medium, the protein content of LB medium was subtracted. Protein concentrations were determined with a Bradford protein assay (section II.6.8).

II.6.3 Discontinuous SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were analysed by SDS-PAGE as described by Laemmli (1970) with modifications by Righetti *et al.* (1990) for discontinuous SDS-PAGE. Protein samples were denatured by heating to 99°C for 5 min in SDS loading dye to denature the proteins. In case of activity gels, the heating was omitted. The polyacrylamide concentration of the gels was chosen according to the expected proteins sizes varying from 10 to 15 % (v/v). Samples were loaded onto the prepared gel which was run at 40 mA until the bromophenol blue reached the lower end. During electrophoresis, proteins were first focussed in the stacking gel and subsequently separated according to their relative molecular mass in the running gel. The size standards employed were High Molecular Weight Marker (MBI Fermentas; St. Leon-Rot; Germany), Precision Plus Protein Standard (Bio-Rad, Munich, Germany) or Protein Molecular Weight Marker (MBI Fermentas; St. Leon-Rot; Germany). For detection of protein activity on an SDS-PAGE (section II.6.6) and for immunochemical detection of the proteins (section II.6.5), the size standard PageRuler Prestained Protein Ladder (MBI Fermentas; St. Leon-Rot; Germany) was used. Gels were stained with Coomassie Brilliant Blue G-250 and destained until the protein bands were clearly visible. For documentation, gels were scanned (ScanMakerX_{12}USI; ScanWizard 5; Microtek; Willich; Germany) and for storage dried using cellophane foil.

II.6.3.1 Solutions and Markers for Discontinuous SDS-PAGE

Stacking gel [6 % (v/v)]	Acrylamide stock solution	500.0	µl
	Buffer for stacking gel	625.0	µl
	H_2O_{deion}	1.375	µl
	APS solution	3.0	µl
	TEMED	30.0	µl
Running gel [10 % (v/v)]	Acrylamide stock solution	1.7	ml
	Buffer for running gel	1.25	ml
	H_2O_{deion}	2.05	ml
	APS solution	5.0	µl
	TEMED	50.0	µl
Running gel [12 % (v/v)]	Acrylamide stock solution	2.0	ml
	Buffer for running gel	1.25	ml
	H_2O_{deion}	1.75	ml
	APS solution	5.0	µl
	TEMED	50.0	µl
Running gel [15 % (v/v)]	Acrylamide stock solution	2.5	ml
	Buffer for running gel	1.25	ml
	H_2O_{deion}	1.25	ml
	APS solution	5.0	µl
	TEMED	50.0	µl
Acrylamide stock solution Rotiphorese® Gel 30 (37.5 : 1) (Roth)	Acrylamide	30.0 % (w/v)	
	N,N'-methylen bisacrylamide	0.8 % (w/v)	
APS solution	Ammonium peroxodisulfate	10.0 % (w/v)	
	dissolved in H_2O_{deion}, store at -20°C		
Buffer for running gel	Tris-HCl (pH 8.8)	1.5	M
	SDS	0.4 % (w/v)	
	dissolved in H_2O_{deion}		
Buffer for stacking gel	Tris-HCl (pH 6.8)	0.5	M
	SDS	0.4 % (w/v)	
	dissolved in H_2O_{deion}		

Electrophoresis buffer	Tris-HCl (pH 8.8)	50.0 mM
	Glycine	385.0 mM
	SDS	0.1 % (w/v)
	dissolved in H_2O_{deion}	
Staining solution	Ethanol	30.0 % (v/v)
	Acetic acid	10.0 % (v/v)
	Coomassie Brilliant Blue G-250	0.25 % (w/v)
	dissolved in H_2O_{deion}	
Destaining solution	Ethanol	30.0 % (v/v)
	Acetic acid	10.0 % (v/v)
	dissolved in H_2O_{deion}	

High Molecular Weight Marker (Sigma-Aldrich; St. Louis; USA)

The marker was composed of proteins with the following approximate M_r:

205,000; 116,250; 97,400; 84,000; 66,000; 55,000; 45,000; 36,000.

PageRuler Prestained Protein Ladder (MBI Fermentas; St. Leon-Rot; Germany)

The marker was composed of proteins with the following approximate M_r:

170,000; 130,000; 100,000; 70,000; 55,000; 40,000; 35,000; 25,000; 15,000; 10,000.

Precision Plus Protein Standard (Bio-Rad; Munich; Germany)

The marker was composed of proteins with the following approximate M_r:

250,000; 150,000; 100,000; 75,000; 50,000; 37,000; 25,000; 20,000; 15,000; 10,000.

Protein Molecular Weight Marker (MBI Fermentas; St. Leon-Rot; Germany)

The marker was composed of proteins with the following approximate M_r:

116,000; 66,200; 45,000; 35,000; 25,000; 18,400; 14,400.

II.6.4 Two Dimensional (2D) Gel Electrophoresis

The two dimensional (2D) gel electrophoresis is a method for the separation of a complex protein mixture. In the first dimension, the separation is based on the isoelectric point of the proteins. This procedure separates proteins due to their charges. In the second dimension, the separation is carried out according to their relative molecular masses (M_r) by SDS-PAGE analysis as described in section II.6.3.

II.6.4.1 Isoelectric Focussing (IEF)

Proteins of 9 ml of cell-free growth medium precipitated (section II.6.1.2) were used for IEF. The dried proteins were dissolved in 83.33 µl of rehydration buffer A and mixed with 41.67 µl of rehydration buffer B. This mixture was filled into the IEF chamber (PROTEAN IEF-Cell; Bio-Rad; Munich; Germany). The pH-gradient strip (7 cm; pH 3 – 10; Bio-Rad) was placed into the chamber with the gel coated side upside down. The dried gel on the strip swelled, and the proteins were soaked into the gel. After 1 h, 2 ml of mineral oil were added to protect the strips from evaporation. The strips were further incubated for 16 h at RT. The IEF was conducted using the following program at RT and constant currency of 50 µA per gel.

Step	Tension	Time	Transition
1	250 V	1 h	rapid
2	500 V	1 h	rapid
3	1000 V	1 h	rapid
4	5000 V	4 h	rapid
5	500 V	Break	rapid

Then, the pH-gradient strips were directly used for SDS-PAGE.

II.6.4.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The pH-gradient strips were incubated for 15 min in equilibration buffer containing 15 mM DTT. Subsequently, an incubation of 15 min was carried out in equilibration buffer containing 150 mM of iodacetamide. All steps occurred at RT.

The pH-gradient strips were placed on the top of the SDS-PAGE gel and fixed with sealing solution. The discontinuous SDS-PAGE was carried out as described in section II.6.3.

II.6.4.3 Solutions for Two Dimensional Gel Electrophoresis

Rehydration solution A	Urea	7.0	M
	Thio urea	2.0	M
	CHAPS	4.0	% (w/v)
	DTT	50.0	mM
	dissolved in H_2O_{deion}, store at -80°C		

Rehydration solution B	Rehydration solution A	2.625	ml
	Ampholyte solution (for pH 3 – 10)	125.0	µl
	Bromophenol blue	350.0	µM
	store at -80°C		
SDS equilibration buffer	Tris-HCl (pH 8.8)	50.0	mM
	Urea	6.0	M
	SDS	2.0	% (w/v)
	Glycerol	35.0	% (w/v)
	Bromophenol blue	350.0	µM
	dissolved in H_2O_{deion}, store at -20°C		
Sealing solution	Agarose	1.0	% (w/v)
	Bromophenol blue	350.0	µM
	dissolved in SDS electrophoresis buffer (section II.6.3)		

II.6.5 Immunochemical Detection of Proteins by Western Blot

For the immunochemical detection, the electrophoretically separated proteins (section II.6.2) were transferred onto a polyvinylidene difluoride (PVDF – pore width 0.45 µm) membrane (Roti®; Roth; Karlsruhe; Germany) using a Trans Blot apparatus (semi dry transfer cell; Bio-Rad; Munich, Germany). Proteins bind to the membrane by polar interaction allowing detection by an enzymatic colour reaction.

After the SDS-PAGE, the unstained gel and two pieces of Whatman paper were equilibrated in Towbin buffer for 15 min. The PVDF membrane was activated in methanol for 15 min and then equilibrated in Towbin buffer. Proteins were blotted onto the membrane for 10 min at 15 V per gel using the semi dry method (Bio-Rad; Munich; Germany). After blotting, proteins were reversibly stained with Ponceau S.

II.6.5.1 Immunochemical Detection of His-Tagged Proteins

For detection of His_6-tagged proteins, the stripped membrane was blocked with blocking buffer H for 1 h. Then, the membrane was incubated with monoclonal mouse anti-His-tag antibody (GE Healthcare; Uppsala; Sweden) for 1 h, washed three times for 10 min with washing solution H and incubated for 45 min with goat anti-mouse IgG antibody conjugated with alkaline phosphatase (Sigma-Aldrich; St. Louis; USA). After four washing steps for 10 min in 1 x PBS-Tween, the detection of the immunoreactive protein was performed using the

NBT/BCIP colour developing method (Nitroblue tetrazolium/5-Brom-4-chlor-3-indolyl phosphate) from Promega (Mannheim; Germany). Therefore, the membrane was equilibrated in alkaline phosphatase buffer (APB) for 10 min. The reaction of the alkaline phosphatase was initialised by adding staining solution to the membrane and stopped by washing in H_2O_{deion}. All steps occurred at RT.

II.6.5.2 Immunochemical Detection of Strep-Tagged Proteins

For the detection of StrepII-tagged proteins, the membrane was blocked with blocking buffer S for 1 h. BSA was used for blocking the membrane because skimmed milk powder contains a lot of biotin which is the antigen of Streptavidin antibody conjugate. After three washing steps for 5 min in PBS-Tween, the membrane was incubated for 10 min in PBS-Tween containing 2 µg of Avidin. The membrane was incubated in this solution with the Streptavidin alkaline phosphatase conjugate (IBA GmbH; Göttingen; Germany) for 1 h. After three washing steps for 1 min in PBS-Tween and two washing steps for 1 min in PBS, the reaction of the alkaline phosphatase was initiated as described in section II.6.5.1. All steps occurred at RT.

II.6.5.3 Antibodies and Antibody Conjugates for Immunochemical Detection

Antibodies and antibody conjugates	Antigen	Company	Dilution
mouse anti His-tag antibody (Primary antibody)	His-tag	GE Healthcare	1 : 2,000 in blocking solution
goat anti-mouse IgG AP conjugated antibody (Secondary antibody)	IgG Fc-specific	Sigma-Aldrich	1 : 5,000 in washing solution
Streptavidin alkaline phosphatase conjugate	Strep-tag	IBA GmbH	1 : 4,000 in PBS-Tween + 2 µg ml^{-1} Avidin

II.6.5.4 Solutions for Immunochemical Detection

Towbin buffer	Tris-HCl (pH 9.5)	25.0	mM
	Glycine	194.0	mM
	Methanol	20.0	% (v/v)
	dissolved in H_2O_{deion}		

10 x PBS (pH 7.4) (phosphate buffered saline)	NaCl	1.37	M
	KCl	27.0	mM
	Na_2HPO_4	50.0	mM
	K_2HPO_4	15.0	mM
	dissolved in H_2O_{deion}		
5 x Ponceau S	Ponceau S	2.0 % (w/v)	
	Trichloroacetic acid	30.0 % (w/v)	
	Sulfo salicylic acid	30.0 % (w/v)	
	dissolved in H_2O_{deion}		
Blocking buffer H	Skimmed milk powder	5.0 % (w/v)	
	Tween 20	0.1 % (w/v)	
	dissolved in 1 x PBS		
Washing buffer H	Skimmed milk powder	0.5 % (w/v)	
	Tween 20	0.1 % (w/v)	
	dissolved in 1 x PBS		
Blocking buffer S	BSA	3.0 % (w/v)	
	Tween 20	0.1 % (w/v)	
	dissolved in 1 x PBS		
1 x PBS/Tween 20	10 x PBS	10.0 % (v/v)	
	Tween 20	0.1 % (v/v)	
	dissolved in H_2O_{deion}		
Alkaline phosphatase buffer (APB)	Tris-HCl (pH 9.5)	100.0	mM
	NaCl	100.0	mM
	$MgCl_2$	5.0	mM
	dissolved in H_2O_{deion}		
Staining solution	APB	10.0	ml
	NBT-solution (100 mg ml^{-1} in 70 % (v/v) dimethylformamide)	33.0	µl
	BCIP-solution (50 mg ml^{-1} in dimethylformamide)	33.0	µl

II.6.6 Preparation of Protein Activity SDS-PAGE Gels

GFP as well as levansucrase were detected on so called protein activity SDS-PAGE gels. GFP was detected due to its green colour observed on the gel whilst the activity of levansucrase was detected by staining its reaction product levan.

The heating of the samples of the intra- or extracellular proteins to 99°C was omitted before loading them onto the SDS-PAGE gel. After electrophoresis as described in section II.6.3, the activity of GFP was visualised on a light table emitting blue light and photographed through a yellow filter using a digital camera (Cyber shot; Sony; Cologne; Germany).

The reaction product levan of the levansucrase was detected in an activity stain after SDS-PAGE. After electrophoresis as described in section II.6.3, the gel was washed three times for 20 min at RT in washing buffer for SDS removal. The following incubation in washing buffer substituted with 292 mM of sucrose for 36 h at 50°C enabled the *in situ* formation of polymers by renatured enzymes. After an additional washing step in 70 % (v/v) ethanol, the gel was incubated in solution Schiff 1 for 1 h at RT. The oxidation by periodic acid (H_5IO_6) activated the polymer for a stain with Schiff reagent (Ferretti *et al.*, 1987). Three washing periods of 20 min at RT in solution Schiff 2 followed the addition of Schiff reagent (Sigma-Aldrich; Munich; Germany). The polymer formation was visualised by purple bands in the gel. Staining was stopped by incubation of the gel in solution Schiff 2. For documentation, gels were scanned (ScanMakerX_{12}USI; ScanWizard 5; Microtek; Willich; Germany). For storage, gels were dried using cellophane foil.

II.6.6.1 Solution for Activity Gels

Washing buffer	Sodium acetate (pH 5.4)	20.0 mM
	$CaCl_2$	340.0 µM
	Triton X-100	0.1 % (v/v)
	dissolved in H_2O_{deion}	
Solution Schiff 1	H_5IO_6	0.7 % (w/v)
	Acetic acid	5.0 % (v/v)
	dissolved in H_2O_{deion}	
Solution Schiff 2	$Na_2O_5S_2$	0.2 % (w/v)
	Acetic acid	5.0 % (v/v)
	dissolved in H_2O_{deion}	

II.6.7 Purification of Recombinantly Produced Proteins by Affinity Chromatography

II.6.7.1 Purification of Intracellular Fusion Proteins

For the purification of the intracellularly produced fusion proteins, cells of 50 ml of shaking flask cultures (section II.6.1) of recombinant *B. megaterium* were harvested by centrifugation (2,600 x g; 15 min; 4°C).

II.6.7.1.1 Purification of His-Tagged Proteins

For purification of the His_6-tagged proteins, cells were suspended in 2.5 ml of binding buffer and disrupted by sonication (SONOPLUS; Bandelin electronics; Berlin; Germany) 3 times for 5 min with 50 % cycle and 70 % power with 5 min breaks. To avoid protein degradation by proteases, protease inhibitor (Complete Mini EDTA-free; Roche; Mannheim; Germany) was used where indicated according to manufacturer's instructions. A Poly-Prep chromatography column (Bio-Rad; Munich; Germany) was packed with 0.2 or 1.0 ml of Chelating Sepharose FF (GE Healthcare; Uppsala; Sweden). The material was first loaded with two column volumes (CV) of 100 mM of $NiSO_4$ and subsequently equilibrated with 5 CV of H_2O_{deion} followed by 5 CV of washing buffer. After an ultra-centrifugation step of the disrupted cells (113,000 x g; 35 min; 4°C) for the separation of cell debris and insoluble proteins from soluble proteins, the cell-free extract containing 1 to 10 mg protein per ml was loaded onto the prepared affinity column. Alternatively, the prior prepared column material was incubated with the soluble proteins in a batch process with smooth shaking for 1 h at 4°C. The sepharose was separated from the solution by a chromatography column. After loading, the column material was washed with 3 CV of washing buffer. Proteins were step eluted at different imidazole concentrations of 50, 100, 200, and 500 mM, respectively, in washing buffer. An aliquot of 20 µl of each fraction was mixed with 5 µl of SDS loading dye for SDS-PAGE analysis (section II.6.3). Moreover, fractions were diluted 1 : 1000 in sodium phosphate buffer (100 mM, pH 7.0) for spectroscopic analysis (section II.6.10).
Harvesting, disruption and ultra-centrifugation were performed at 4°C while all purification steps were performed at RT. Elution fractions were stored at 4°C for further analysis.

II.6.7.1.2 Solution for Purification of His-Tagged Proteins

Binding buffer (pH 7.0)	Na_2HPO_4	100.0 mM
	NaH_2PO_4	100.0 mM
	dissolved in H_2O_{deion}	
Washing buffer (pH 7.0)	Na_2HPO_4	100.0 mM
	NaH_2PO_4	100.0 mM
	Imidazole	7.0 mM
	dissolved in H_2O_{deion}	
Elution buffer (pH 7.0)	Na_2HPO_4	100.0 mM
	NaH_2PO_4	100.0 mM
	Imidazole	50.0 – 500.0 mM
	dissolved in H_2O_{deion}	

II.6.7.1.3 Purification of Strep-Tagged Proteins

For purification of the StrepII-tagged proteins, cells were suspended in 2.5 ml of buffer W and the soluble proteins were harvested like described in section II.6.7.1.1. The cell-free extract containing the soluble protein fraction was loaded onto a 0.2 or 1.0 ml Strep-Tactin affinity column (Strep-Tactin Superflow; IBA GmbH; Göttingen; Germany). This method was performed according to the manufacturer's instructions (SIGMA-Genosys; Haverhill; United Kingdom). After loading, the column material was washed 5 times with 1 CV of buffer W followed by 6 elution steps with half a CV of buffer E. An aliquot of 20 µl of each fraction was mixed with 5 µl of SDS loading dye for SDS-PAGE analysis (section II.6.3). Moreover, fractions were diluted 1 : 1000 in sodium phosphate buffer (100 mM, pH 7.0) for spectroscopic analysis (section II.6.10).

Harvesting, disruption and ultra-centrifugation were performed at 4°C while all purification steps were performed at RT. Elution fractions were stored at 4°C for further analysis.

II.6.7.1.4 Solution for Purification of Strep-Tagged Proteins

Buffer W (pH 8.0)	Tris-HCl	100.0 mM
	EDTA	1.0 mM
	dissolved in H_2O_{deion}	

Buffer E (pH 8.0)	Tris-HCl	100.0 mM
	EDTA	1.0 mM
	Desthiobiotin	2.5 mM
	dissolved in H_2O_{deion}	

II.6.7.2 Purification of Extracellular Fusion Proteins

For the purification of the recombinantly produced and secreted levansucrase (StrepLevΔ773 or LevΔ773His), cells of 100 ml to 500 ml shaking flask cultures (section II.6.1) of recombinant *B. megaterium* were harvested by centrifugation (2,600 x g; 15 min; 4°C) to obtain the cell-free growth medium. To remove the remaining cells, the supernatant was filtrated (Millipore; Billerica; USA; pore width of 0.2 µm). For large scale filtration (2 litre of growth medium), the supernatant was filtrated using a cross-flow filtration system (section II.6.2).

For LevΔ773His purification from the not concentrated cell-free growth medium two alternative purification strategies were used.

(I) Chelating Sepharose FF (GE Healthcare; Uppsala; Sweden) was loaded with $NiSO_4$ as described in section II.6.7.1.1. 700 µl of this Ni-loaded Chelating Sepharose were incubated with 50 ml of cell-free growth medium for 1 h under smooth shaking at RT. The sepharose was separated from the growth medium using a Poly-Prep chromatography column (Bio-Rad; Munich; Germany). The column material was washed with 3 CV of binding buffer. Proteins were step eluted with 1 CV of elution buffer supplemented with 200 mM imidazole and 1 mM β-mercaptoethanol.

(II) 600 µl of MagneHis^{TM} Ni-Particles (Promega; Madison; USA) were incubated in 50 ml of cell-free growth medium for 1 h under smooth shaking at RT. The beads were separated from the growth medium using a strong magnet and washed three times with 1 ml of binding buffer. Proteins were step eluted at different imidazole concentrations of 100 and 500 mM and 1 mM β-mercaptoethanol in 1 ml of binding buffer.

For purification of the LevΔ773His from the concentrated cell-free growth medium (section II.6.2), a chromatography column was packed with 500 µl of Ni-loaded Chelating Sepharose. After equilibration with binding buffer, 30 ml of 25 times concentrated medium containing 255 µg protein ml^{-1} and 7 mM imidazole were loaded onto the column. The column material was washed with 3 CV of binding buffer. Finally, proteins were step eluted (1 CV per step)

with 3 times 100 mM followed by 2 times 500 mM imidazole and 1 mM of β-mercaptoethanol in binding buffer.

StrepLevΔ773 was purified from the cell-free growth medium using a Strep-Tactin sepharose affinity column (IBA GmbH; Göttingen; Germany). 1 ml of Strep-Tactin affinity material was incubated with 50 ml of not concentrated cell-free growth medium for 1 h under smooth shaking at RT in a batch process. The material was separated from the growth medium using a Poly-Prep chromatography column (Bio-Rad; Munich; Germany). Alternatively, 50 ml of not concentrated cell-free growth medium were running over an 1 ml Strep-Tactin affinity column. After washing 5 times with 1 CV of buffer W, proteins were step eluted using 5 times half a CV of buffer E per step.

For purification of StrepLevΔ773 from the concentrated cell-free growth medium, the ammonium sulphate precipitated extracellular proteins (see II.6.1.2) of 30 ml cell-free growth medium were solubilised in 3 ml of buffer W and loaded onto an 1 ml Strep-Tactin affinity column. After washing 5 times with 1 CV of buffer W, proteins were step eluted using 5 times half a CV of buffer E per step.

All purification steps were performed at RT. All eluted fractions were stored at 4°C for further analysis. An aliquot of 20 µl of each fraction were mixed with 5 µl of SDS loading dye and were analysed by SDS-PAGE (section II.6.3).

II.6.7.2.1 Solution for Purification of Extracellular Fusion Proteins

Binding buffer (pH 7.0)	NaCl	50.0 mM
	Na_2HPO_4	100.0 mM
	NaH_2PO_4	100.0 mM
	dissolved in H_2O_{deion}	
Elution buffer (pH 7.0)	Na_2HPO_4	100.0 mM
	NaH_2PO_4	100.0 mM
	Imidazole	100.0 – 500.0 mM
	β-mercaptoethanol	1.0 µM
	dissolved in H_2O_{deion}	
Buffer W (pH 8.0)	Tris-HCl	100.0 mM
	EDTA	1.0 mM
	dissolved in H_2O_{deion}	

Buffer E (pH 8.0)	Tris-HCl	100.0 mM
	EDTA	1.0 mM
	Desthiobiotin	2.5 mM
	β-mercaptoethanol	1.0 mM
	dissolved in H_2O_{deion}	

II.6.8 Determination of Protein Concentration

Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad; Munich; Germany) following the manufacturer's instructions for the low-concentration assay (1.25 – 25 µg of protein per ml). The assay is based on the colorimetric method developed by Bradford (1976). Bovine serum albumin (BSA; Perbio; Rockford; USA) was used as standard. For standard and sample dilution, the same buffers were used.

II.6.9 Protease Digestion for the Removal of Affinity Tags

Fusion tags of the purified recombinant proteins (section II.6.7.1) were eliminated using the peptidase cleavage sites for TEV (tobacco etch virus) (personal gift; S.; Helmholtz Centre for Infection Research; Braunschweig; Germany) or the Factor Xa protease (QIAgen; Hilden; Germany). Therefore, 0.89 µg of His_6-tagged TEV-protease were incubated with 10.8 µg of fusion protein in reaction buffer T in a total volume of 20 µl overnight at 20°C. 0.2 µg of Factor Xa protease were incubated with 6.3 µg of fusion protein in reaction buffer X in a reaction volume of 20 µl overnight at 23°C. Successful cleavage was analysed by SDS-PAGE (section II.6.3).

II.6.9.1 Reaction Buffers for Protease Digestion

Reaction buffer T (pH 8.0)	Tris-HCl	10.0 mM
	NaCl	500.0 mM
	DTT	1.0 mM
	dissolved in H_2O_{deion}	
Reaction buffer X (pH 8.0)	Tris-HCl	100.0 mM
	NaCl	100.0 mM
	$CaCl_2$	2.0 mM
	dissolved in H_2O_{deion}	

II.6.10 Fluorescence Measurements of Green Fluorescent Protein (GFP)

For qualitative GFP-detection in an activity gel (section II.6.6) and for detection of GFP-producing cells on LB medium agar plates, GFP was visualised on a light table emitting blue light and photographed through a yellow filter using a digital camera (Cyber shot; Sony; Cologne; Germany).

For quantification, the amount of GFP was measured via its fluorescence (Luminescence Spectrometer LS50B; PerkinElmer; Boston; USA). Samples were excited at 475 nm and fluorescence emission was detected at 512 nm. For the determination of GFP production over the time course of cultivation, cells were harvested at different time points after induction of gene expression by centrifugation (14,000 x g; 10 min; 4°C) and diluted in sodium phosphate buffer (100 mM, pH 7.0) to a final concentration of 0.5 x 10^9 cells per ml. Furthermore, soluble protein fractions after ultra-centrifugation and flow-through fractions of affinity chromatography were diluted 1 : 500 in sodium phosphate buffer (100 mM, pH 7.0) prior measuring. Wash and elution fractions of protein purification were diluted 1 : 1,000 in sodium phosphate buffer (100 mM, pH 7.0) prior analysis.

The relative fluorescence of different amounts of purified Strep-GFP was determined (section II.6.8) and a linear correlation was deduced to:

$$GFP\ [mg\ ml^{-1}] = \text{relative emission maxima} \times 3.42 \times 10^{-6} \times \text{dilution factor}$$

II.6.11 Determination of Levansucrase Activity

The levansucrase enzyme activity of various preparations described in this investigation was measured via the release of fructose and glucose from sucrose. The total amount of fructose and glucose was determined using dinitrosalicylic acid as described in the D.N.S. method (Sumer and Howell, 1935). Using this method, the observed rate of total fructose and glucose formation is the sum of the rate of released glucose (v_G) and the rate of released fructose (v_F).

Van Hijum *et al.* (2004) reported that the recombinant levansucrase LevΔ773MycHis purified from *E. coli* and incubated with sucrose follows Michaelis-Menten kinetics. For the release of glucose (G) and fructose (F) a K_M^G of 9.7 mM, a k_{cat}^G of 147 s^{-1}, a K_M^F of 11.3 mM and a k_{cat}^F of 117 s^{-1} were determined. In the steady state, the substrate concentration [S] was assumed to be [S_0], which was found about 100 mM sucrose. From the rate of released reducing sugars ($v_G + v_F$), the rate of glucose (v_G) release was calculated using the following formula (for LevΔ773):

$$\frac{v_G}{v_G + v_F} = \frac{\frac{V^G_{\max} \cdot [S]}{K^G_M + [S]}}{\frac{V^G_{\max} \cdot [S]}{K^G_M + [S]} + \frac{V^F_{\max} \cdot [S]}{K^F_M + [S]}} = \frac{\frac{k^G_{cat} \cdot [S]}{K^G_M + [S]}}{\frac{k^G_{cat} \cdot [S]}{K^G_M + [S]} + \frac{k^F_{cat} \cdot [S]}{K^F_M + [S]}} = 0.560$$

Since the levansucrase SacB from *B. megaterium* has not been characterised so far, no kinetic parameters are existing.

All D.N.S. assays were performed at 37°C to avoid abiotic hydrolysis of sucrose (Ozimek *et al.*, 2004). Levansucrase containing solutions were incubated in reaction buffer (van Hijum *et al.*, 2004) in a total volume of 1.5 ml and at 1,000 rpm (Thermomixer compact, Eppendorf, Germany). Growth medium containing levansucrase was tested after 50-fold dilution. Concentrated growth medium and chromatographic fractions were tested 125-fold diluted in the reaction buffer. Samples were taken at different time points after the reaction was started. To stop the reaction, samples were diluted 1 : 1 with D.N.S. reagent and stored in the dark. In parallel, standard solutions containing 0 – 2 mg l^{-1} glucose were mixed 1 : 1 with D.N.S. reagent. Samples and standards were denatured at 99°C for 5 min and immediately cooled down on ice. For quantification, samples and standards were diluted 1 : 6 with H_2O_{deion} and the OD was measured at 540 nm.

II.6.11.1 Solution for Determination of Levansucrase Activity

Reaction buffer (pH 5.4)	$Na(C_2H_3O_2)$	25.0 mM
	Sucrose	100.0 mM
	$CaCl_2$	1.0 mM
	NaN_3	0.7 µM
	dissolved in H_2O_{deion}	
D.N.S. reagent	3,5-dinitrosalicylic acid	10.0 g l^{-1}
	$KNaC_4H_4O_6$	300.0 g l^{-1}
	NaOH	16.0 g l^{-1}
	dissolved in H_2O_{deion}	

II.6.12 Determination of Penicillin G Acylase (PGA) Activity

Instead of the original target substrate, penicillin G, the synthetic substrate 6-Nitro-3-phenylacetamido-benzoic acid (NIPAB) was used for determination of penicillin G acylase (PGA) activity (Kutzbach and Rauenbusch, 1974). Substrate conversion was followed by spectrophotometric measurements based on the release of the chromophore of the synthetic substrate NIPAB.

For quantification of PGA concentration in the cell-free growth medium, 900 µl of cell-free PGA containing solution were mixed with 100 µl of NIPAB reagent. Directly after starting the reaction, the change of absorption was quantified continuously at an OD of 405 nm at 25°C for 60 s. Activity of penicillin G acylase was calculated using the following formula:

$$\text{activity}\left[\frac{\text{Units}}{\text{ml}}\right] = \frac{\Delta A \cdot V_R}{\alpha \cdot d \cdot t \cdot V_S}$$

ΔA: Change of absorption

α: Differential molar absorption (8.98 ml µmol^{-1} cm^{-1})

d: Gap width of cuvette

t: Reaction time

V_R: Reaction volume (1 ml)

V_S: Sample volume (100 µl)

II.6.12.1 Solution for Determination of Penicillin G Acylase Activity

Sodium phosphate buffer (pH 7.5)	Na_2HPO_4	100.0	mM
	NaH_2PO_4	100.0	mM
	dissolved in H_2O_{deion}		
NIPAB reagent	2-nitro-5-[(phenylacetyl)amino]-benzoic acid	60.0	mg
	Sodium phosphate buffer (pH 7.5)	50.0	% (v/v)
	dissolved in H_2O_{deion}		

II.6.13 Bioreactor Cultivation

The bioreactor used in this study was a Biostat B2 (B. Braun; Melsungen; Germany) with 2 litres of working volume connected to an exhaust gas analysis unit (Maihak; Hamburg; Germany). The bioreactor control unit was connected to a computer running the MFCS

software (B. Braun; Melsungen; Germany). In addition, feed and base reservoirs were stored on scales connected to the control computer. The pH control of the culture was achieved by the regulated addition of either 5 M of NaOH or 1 M of H_3PO_4. *B. megaterium* WH323 carrying pRBBm56 coding for GFP-Strep was grown in A5 medium at 37°C (Malten *et al.*, 2005a) initially in a batch phase with 30 g l^{-1} glucose in the first and 4 g l^{-1} in the second run. At the end of the batch phase observed by a rise in the concentration of dissolved oxygen, an exponential feeding profile was started with a set growth rate (μ_{set}) of 0.12 h^{-1} in the first and 0.14 h^{-1} in the second run. During fed-batch cultivation, 10 ml of trace element solution (section II.3.1) and 1.2 g of $MgSO_4$ were added discontinuously. Recombinant gene expression was induced by the addition of 0.5 % (w/v) xylose after 11.2 h (μ_{set} of 0.12 h^{-1}) and 31.7 h (μ_{set} of 0.14 h^{-1}), respectively. Samples for measurements of biomass, metabolite and GFP concentration were taken at different time points before and after induction of GFP production.

II.6.13.1 Analytical Procedures

Concentrations of glucose and formed metabolites were determined using HPLC analysis on an Aminex HPX-87H column (Bio-Rad; Hercules; USA). 10 mM of H_2SO_4 at a flow rate of 600 µl min^{-1} and 60°C was used as mobile phase in order to separate xylose from pyruvate. The data from the exhaust analysis in conjunction with metabolite and biomass data were used for carbon balancing. All fermentations showed closed carbon balances with values between 98 and 117 %. The amount of GFP was evaluated in microtiter plates using a Fluoroskan Ascent reader (Thermo; Waltham; USA). After calibration of the system using purified GFP-Strep (section II.6.7.1), 3 x 10^9 cells were harvested, suspended in 300 µl of sodium phosphate buffer (100 mM, pH 7.0) and diluted 1 : 10. 100 µl of these dilutions were measured in 4 replicates with an excitation at 485 nm and an emission at 535 nm.

II.6.14 Fluorescent Staining and Flow Cytometry

Viability stains of *B. megaterium* WH323 carrying pRBBm56 during the fermentation process were performed using the propidium iodide (PI) based Cell Viability Kit (BD Bioscience; San Jose; USA). Therefore, cells from 10 µl of fed-batch sample (section II.6.13) were harvested by centrifugation (14,000 x g; 1 min; 4°C) and suspended in ice-cold 1 x PBS (pH 7.0) to a final concentration of (0.5 – 5) x 10^8 cells per ml. As negative control, 10 min prior to centrifugation ethanol was added to 10 µl of a fed-batch sample to a final concentration of

70 % in order to obtain cell killing. 500 µl of dilutions were stained with 2 µl of PI. Flow cytometry analysis was performed using a FACSCalibur (Benton Dickinson; Erembodegen-Aalst; Belgium) and analysed using the according CellQuestPro and Summit® software (DakoCytomation; Fort Collins; USA). Side (SSC) and frontal scatter (FSC) triggers were adjusted to show the whole cell population. Green fluorescence due to GFP production was collected in the FL1 channel (520 ± 20 nm) while red fluorescence due to PI in the FL3 channel (> 610 nm). For all measurements, triggering of FL1 and FL3 was kept constant. A gate comprising 92 to 94 % of all events was first constructed on the FCS and SSC dot blot comprising all dead cells (for more details see section III.1.7.2, **Fig. 21**). All gated events were depicted in combined red (FL3) and green (FL1) fluorescence dot blots and gated by a quadrant in order to separate the subpopulations.

III RESULTS AND DISCUSSION

III.1 INTRACELLULAR PRODUCTION OF RECOMBINANT PROTEINS IN *BACILLUS MEGATERIUM*

III.1.1 Vectors for the Intracellular Production of Recombinant Fusion Proteins Carrying Small Affinity Tags

Over several decades, different vector systems for recombinant gene expression in *E. coli* were developed. Beside the origin of replication, the selection marker gene and the multiple cloning site (MCS), expression vectors often include coding sequences for so called affinity tags upstream or downstream of the MCS. These affinity tags simplify the later purification of the recombinant gene product. For the production of recombinant proteins in *B. megaterium*, the xylose-inducible promoter system (Rygus *et al.*, 1991) was previously used for recombinant gene expression (Barg *et al.*, 2005; Burger *et al.*, 2003; Malten *et al.*, 2005a; Rygus and Hillen, 1991; Yang *et al.*, 2006).

In this thesis a series of *B. megaterium* expression vectors for the intra- and extracellular production of recombinant proteins carrying His_6- or/and StrepII-tags for subsequent affinity chromatography based purification were designed and constructed. In a first step based on the previously in our laboratory constructed shuttle vector pMM1520 (Malten *et al.*, 2005a), the vector pSTOP1522 was developed (**Fig. 13**). Its additional *Bsr*GI restriction site downstream of the ribosome binding site (RBS) and upstream of the MCS allows for the cloning of target genes keeping their original start codon directly at the position of the transcriptional start of the *xylA* gene. The stop codon followed by the *Nru*I restriction site closes the open reading frame of the MCS. Therefore, target genes may be cloned without their own original stop codon. This shuttle vector contains elements for replication and selection in *E. coli* (origin of replication (*colE1*) and *β-lactamase* gene) and for replication, selection and xylose-inducible gene expression in *B. megaterium* (origin of replication (*oriU*) and *repU* gene, tetracycline resistance gene, xylose operon repressor gene (*xylR*) and the xylose-inducible promoter P_{xylA} sequence followed by the first 15 bp of the xylose isomerase gene (*xylA*)).

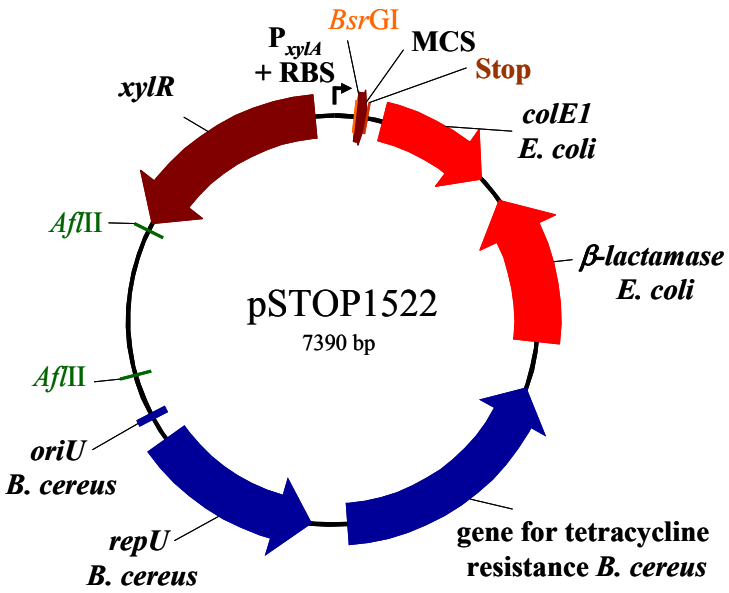


Figure 13: Structure of the *B. megaterium* expression vector pSTOP1522. pSTOP1522 is based on the shuttle vector pMM1520 (Malten *et al.*, 2005a). The additional *Bsr*GI site between ribosome binding site (RBS) and multiple cloning site (MCS) is marked in orange, the stop codon downstream of the MCS in brown. Elements for xylose-inducible recombinant gene expression in *B. megaterium* are the xylose-inducible promoter P_{xylA} and the gene encoding the xylose repressor (*xylR*, indicated in brown). Elements for plasmid replication in *Bacillus sp.* are *oriU* representing the origin of plasmid replication, *repU*, a gene essential for plasmid replication and the tetracycline resistance gene (all indicated in blue). Elements for plasmid replication in *E. coli* are the origin of replication *colE1* and the *β-lactamase* gene, responsible for the ampicillin resistance (both indicated in red). The two *Afl*II endonuclease restriction sites (in green) were used to create the vector series 1622 reduced in size by 855 bp.

Based on pSTOP1522, several *B. megaterium* vectors allowing the production of target proteins with fusions to different affinity tags were constructed. For the removal of genetic ballast, the vector was liberated from an 855 bp *Afl*II fragment comprising a partial and inactive tetracycline resistance gene. This resulted in a family of plasmids all carrying the number 1622 within their names (**Fig. 13+14**).

Two different affinity tags, the StrepII-tag (Schmidt *et al.*, 1996) and the polyhistidine (His_6-) tag (Porath *et al.*, 1975), were chosen as N- or C-terminal fusion partners for target proteins. They are encoded on the novel expression plasmids either upstream or downstream of the 63 bp open reading frame (ORF) including the MCS (**Fig. 14**).

For some of the resulting fusion proteins the corresponding N-terminal affinity tag may be removed via protease digestion, either by the tobacco etch virus (TEV) (Kapust and Waugh, 2000) or the Factor Xa protease (Nagai and Thogersen, 1984). Both proteases are suited for the generation of an N-terminus with not more than one additional amino acid.

For the insertion of all these short dsDNA fragments, the method of synthetic oligonucleotides cloning was employed.

The StrepII-tag, commercialised by the IBA GmbH (Göttingen; Germany), is a peptide with the amino acid sequence WSHPQFEK which has a strong affinity to Strep-Tactin (Schmidt and Skerra, 1993). It can be either placed in the middle of a protein or at its N- and C-terminus, respectively (Schmidt *et al.*, 1996). StrepII-tag fusion proteins are readily purified from a cell-free extract using a Strep-Tactin affinity column. The natural ligand biotin is used for the elution of the fusion proteins from the column. Advantages of this system are the mild elution conditions and the simple recycling of the affinity support material. Novel StrepII-tag encoding plasmids constructed in this thesis are pN-STREP-Xa1622, pN-STREP-TEV1622 and pC-STREP1622 (**Fig. 14B**). Vector pN-STREP-Xa1622, based on pSTREP1522, is useful for the recombinant production of fusion proteins carrying an N-terminal StrepII-tag followed by a protease cleavage site for the Factor Xa, whereas pN-STREP-TEV1622 encodes an N-terminally fused StrepII-tag followed by a 3 amino acid spacer region and a TEV protease cleavage site upstream of the MCS. The plasmid pC-STREP1622 encodes the StrepII-tag downstream of the MCS for the production and purification of C-terminal StrepII-tagged target proteins.

Protein purification by a His-tag (Hochuli *et al.*, 1987) is the most common method for the purification of recombinant proteins. Six adjacent histidine residues confer a strong interaction between the tag and the nickel ion, immobilised on a nitrilotriacetic acid (NTA) agarose resin. The competitive ligand imidazole is used for the mild elution of the fusion protein from the affinity column. His-tags can be placed at the N- or C-terminus of proteins. Hence, novel His_6-tag encoding plasmids are pN-HIS-TEV1622 and pC-HIS1622. pN-HIS-TEV1622 encodes an N-terminally fused His_6-tag followed by a 3 amino acid spacer and a TEV protease cleavage site upstream of the MCS. In pC-HIS1622, which based on pHIS1522, the sequence of the His_6-tag is located downstream of the MCS. This vector is useful for the fusion of proteins to a C-terminal His_6-tag (**Fig. 14B**).

The synthetic oligonucleotides for the construction of the vectors encoding N-terminal affinity tags were cloned in frame downstream of the first 15 bases of *xylA'* in pSTOP1522 or pSTOP1622. The oligonucleotides encoding the C-terminal affinity tags were cloned in frame to the stop codon maintaining the restriction sites *Bgl*II, *Bsp*EI, *Eco*ICRI, *Sac*I, *Xma*I, *Sma*I, *Bam*HI, *Acc*65I, *Kpn*I, *Ngo*MIV, *Sph*I, and *Nae*I of the MCS (**Fig. 14A+B**). Therefore, the identical structure of the MCS of these plasmids allows in parallel cloning and fusion of the target gene to the coding sequences of the different affinity tags.

Coding sequences of the affinity tags integrated into pN-STREP-Xa1622, pC-STREP1622 and pC-HIS1622 are based on DNA-sequences of pASK-IBA plasmids (IBA GmbH; Göttingen; Germany). This DNA showed codon adaptation indices (CAIs) for *B. megaterium* of 0.57, 0.52, and 0.4, respectively. In pN-STREP-TEV1622 and pN-HIS-TEV1622 the codon usage of the DNA encoding the StrepII-tag, the spacer and the TEV protease cleavage site was adapted to *B. megaterium* with the help of the program JCat (http://www.jcat.de) (Grote *et al.*, 2005). This resulted in a CAI of 0.9 for pN-STREP-TEV1622 and 0.94 for pN-HIS-TEV1622.

To obtain the original N-terminus of the target protein after protease cleavage, the restriction site *Nar*I (indicated as * in **Fig. 14B**), which is located downstream of the coding sequence of the protease cleavage sites, was introduced when cloning the synthetic oligonucleotides. Using the TEV protease, which is reported to be more specific than the Factor Xa protease (Terpe, 2003), the glycine residue of the ENLYFQG recognition site is left at the N-terminus of the recombinant protein. For keeping its original N-terminus, it was reported that the glycine residue could also be substituted by other amino acids as S, A, M, C, N, H, Y, K, D, Q, and F leaving more than 90 % protease digestion efficiency (Kapust *et al.*, 2002). After cleavage by Factor Xa protease with the recognition site of IEGR, no additional amino acid will be left at the N-terminus. A defined and original N-terminus is often of importance if further *e.g.* crystallographic studies are desired.

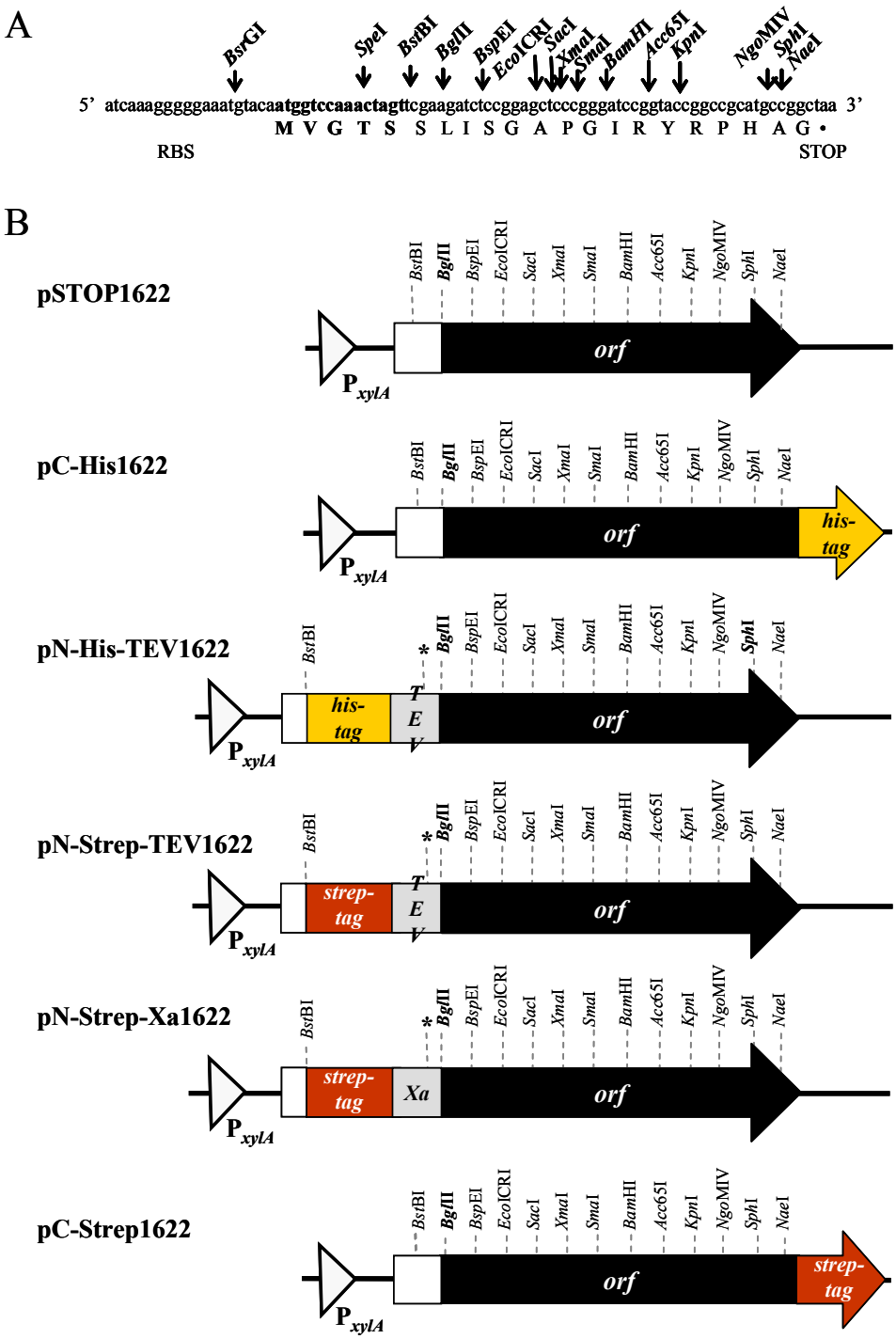


Figure 14: Series of expression plasmids for the intracellular production of tagged proteins in *B. megaterium*. All plasmids of the series 1622 are based on the shuttle vector pSTOP1522. **(A)** DNA sequence of ribosome binding site (RBS) and multiple cloning site (MCS) of the expression plasmid pSTOP1622. The coding sequence of an open reading frame (ORF) comprising the MCS is given. The sequence of the first five amino acids of XylA is marked in bold. All restriction sites of the MCS are indicated. **(B)** Scheme of expression plasmids based on pSTOP1622. All expression plasmids shown allow parallel cloning of genes of interest into the identical MCS from *Bgl*II (marked in bold) to *Nae*I. Restriction site *Nar*I is indicated by a star (*). P_{xylA}: promoter of *xylA*; TEV: tobacco etch virus protease cleavage site; Xa: Factor Xa protease cleavage site.

III.1.2 Production of Different Affinity Tag Carrying GFP

III.1.2.1 GFP as Model Protein for the Intracellular Recombinant Protein Production by *Bacillus megaterium*

For the intracellular recombinant protein production by *B. megaterium*, the variant GFPmut1 of the green fluorescent protein (GFP) from the jellyfish *A. victoria* was chosen as model protein. GFPmut1, with amino acid exchanges in the chromophore forming region (F64L, S65T) (here referred to as GFP), was originally optimised for promoter studies since it can already be detected 8 min after the *gfp* gene expression (Cormack *et al.*, 1996). The use of GFP had several advantages in the course of this work. First, successful cloning of *gfp* is already detectable in *E. coli* via their green colour due to a leakiness of the *xylA* promoter in the cloning host and the resulting GFP synthesis. Further, the *gfp* gene has a CAI of 0.45 for *B. megaterium*. Therefore, it was well suited for expression in *B. megaterium*. Third, due to a linear correlation between the fluorescence intensity and the protein amount (Scholz *et al.*, 2000), intracellular formed GFP can be quantified via fluorescence spectroscopy without cell disruption. This fact allows plate assays (**Fig. 15**) and online analysis (Reischer et al., 2004). The former would be of great interest, *e.g.* for high throughput screening, while the latter may be useful in controlled fermentations to immediately react on changes in the production process.

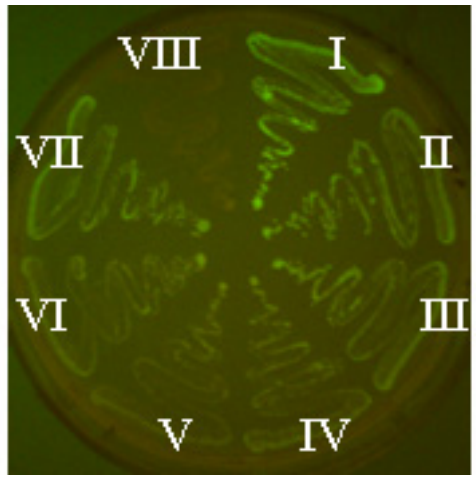


Figure 15: GFP-producing *B. megaterium* cells. *B. megaterium* cells carrying different expression plasmids with coding sequences for GFP (I-VII) or a target geneless plasmid (VIII) as negative control were streaked out onto an LB medium agar plate containing 10 µg ml^{-1} of tetracycline and 0.5 % (w/v) xylose. The *gfp* gene expression was induced via xylose. The plate was incubated overnight at 30°C. Intracellularly produced GFP was visualised under blue light using a yellow camera lens filter.

The DNA sequence encoding the modified GFPmut1 (*gfp*) lacking its original start and stop codon was amplified using pMUTIN-GFP+ (BGSC; USA) as template. It was cloned in frame into the MCS of pSTOP1622. The resulting vector was named pRBBm53.

III.1.2.2 Calculation of Intracellular GFP Amounts Using Purified GFP

Since the quantity of GFP and its fluorescence intensity are proportional (Scholz *et al.*, 2000), purified GFP-Strep (section III.1.4) was used to define the correlation between the amount of GFP-Strep and its fluorescence. The amount of purified GFP-Strep was determined by the Bradford protein assay (Bio-Rad; Munich; Germany) and the corresponding fluorescence emission maxima of these samples were measured spectroscopically. The linear correlation was deduced to:

$$\text{GFP [mg ml}^{-1}\text{]} = \text{relative emission maxima x } 3.42 \text{ x } 10^{-6} \text{ x dilution factor}$$

III.1.2.3 Production of GFP in Various *Bacillus megaterium* Strains at Different Growth Temperatures

After protoplast transformation of *B. megaterium* mutant strains WH320 (Rygus *et al.*, 1991), WH323 (Rygus and Hillen, 1992), and MS941 (Wittchen and Meinhardt, 1995) with pRBBm53 (encoding GFP), all novel plasmid carrying strains were grown aerobically in LB medium at 30°C or 37°C. Negative controls were outlined strains carrying the target geneless pSTOP1622. All mutant strains derive from *B. megaterium* strain DSM319. Strain WH320 is a chemical mutant with no detectable β-galactosidase (*lacZ*) activity, but may also contain further unknown mutations. In WH323, a derivative of WH320, the *xylA* gene was knocked out by the selective insertion of the *E. coli lacZ* gene encoding β-galactosidase (*xylA1-spoVG-lacZ*). In *B. megaterium* MS941, the gene encoding the main extracellular protease NprM was deleted (*ΔnprM*).

The recombinant gene expression was induced by the addition of 0.5 % (w/v) xylose at an OD_{578nm} of 0.4. Samples for determination of OD_{578nm} and GFP amounts were taken 0.5, 2, 3, 4.5, 6, and 7.5 h after induction of the *gfp* gene expression. For quantification of GFP, cells were separated from the growth medium by centrifugation and diluted in sodium phosphate buffer to a final concentration of 10^9 cells per ml. The spectroscopic analysis of the strains carrying pSTOP1622 as negative control failed to detect fluorescence at all analysed time points. The analysis of various employed *B. megaterium* strains carrying pRBBm53 grown aerobically at 37°C revealed differences in the amount of produced GFP (**Tab. 8**). For determination of the intracellular GFP amount it was known that the affinity tags do not influence fluorescence. This is shown in section III.1.3. The introduced correlation (section III.1.2.2) based on GFP-Strep was used for the calculation of the intracellular recombinant GFP amounts. In MS941 carrying pRBBm53, a maximal GFP concentration of 3.2 mg per g

cell dry weight (CDW) was determined 3 h after induction of the *gfp* gene expression. For WH320 and WH323 both carrying pRBBm53, the highest production of GFP was observed with 6.5 mg g_{CDW}^{-1} and 6.6 mg g_{CDW}^{-1}, respectively, 4.5 h after induction of the *gfp* gene expression. Hence, WH323 and WH320 produced 2.1-fold more GFP compared to MS941. *B. megaterium* strain WH323 was chosen for further GFP production experiments. Due to lacking the xylose isomerase XylA, cells do not consume the inducer xylose and are thus better applicable in xylose-mediated recombinant protein production in glucose limited high cell density cultivation experiments (section III.7). Decreasing the growth temperature from 37°C to 30°C led to an 1.4-fold increase in the GFP amount from 6.6 to 9.3 mg GFP per g_{CDW} 6 h after induction of the *gfp* gene expression (**Tab. 8**). The volumetric amount of GFP only increased from 11.3 to 12.0 mg per litre growth medium which is due to the decreased growth from a CDW of 1.73 g l^{-1} to 1.28 g l^{-1}.

Table 8: Comparison of different plasmid carrying *B. megaterium* strains producing GFP. *B. megaterium* strains MS941 carrying pRBBm53 (encoding GFP), WH320 carrying pRBBm53 and WH323 carrying pRBBm53 were grown aerobically in 50 ml of LB medium in shaking flasks at 30°C or 37°C. The *gfp* gene expression was induced at an OD_{578nm} of 0.4 by the addition of 0.5 % (w/v) xylose. Samples were taken at different time points. Amounts of intracellular produced GFP were measured spectroscopically. Time points with maximal values of GFP production are indicated.

B. megaterium strains + plasmids	Time after induction [h]	Expression temperature [°C]	OD_{578nm} [-]	CDW [g l^{-1}]	GFP [mg l^{-1}]	GFP [mg g_{CDW}^{-1}]
MS941-pRBBm53	3.0	37	5.5	1.84	5.9 +/- 0.3	3.2 +/- 0.2
WH320-pRBBm53	4.5	37	4.8	1.55	10.1 +/- 0.5	6.5 +/- 0.3
WH323-pRBBm53	4.5	37	5.0	1.73	11.3 +/- 0.6	6.6 +/- 0.3
WH323-pRBBm53	6.0	30	3.7	1.28	12.0 +/- 0.6	9.3 +/- 0.5

III.1.3 Production of His_6- and StrepII-Tagged GFP in *Bacillus megaterium*

After successful production of GFP in *B. megaterium* cells, the *gfp* gene of pMUTIN-GFP+ was amplified without its start and stop codon and cloned in parallel into the expression vectors pN-STREP-TEV1622, pN-STREP-Xa1622, pN-HIS-TEV1622, pC-HIS1622, and pC-STREP1622. New constructed vectors were named pRBBm54 (encoding Strep-TEV-GFP), pRBBm36 (encoding Strep-Xa-GFP), pRBBm55 (encoding His-TEV-GFP), pRBBm35 (encoding GFP-His), and pRBBm56 (encoding GFP-Strep), respectively. After protoplast transformation of *B. megaterium* WH323 with these expression vectors, cells were grown

aerobically in 50 ml of LB medium at 30°C. The *gfp* gene expression was induced by the addition of 0.5 % (w/v) xylose at an OD_{578nm} of 0.4. Growth was followed by OD_{578nm} measurements. Samples for protein analysis via SDS-PAGE, Western blot and fluorescence spectroscopy were taken 0.5, 2, 3, 4.5, 6, and 7.5 h after induction of the *gfp* gene expression.

All tested *B. megaterium* strains carrying the various plasmids showed comparable growth profiles and reached stationary phase about 6 h after induction of the *gfp* gene expression with a final OD_{578nm} of around 4.0. SDS-PAGE analysis (**Fig. 16A+B**) and spectroscopic measurements (**Fig. 16C**) of samples at indicated time points showed significant GFP-production in all plasmid carrying strains except in the negative control carrying the target geneless plasmid pMM1622. Maximal GFP-production was observed 6 h after induction of the *gfp* gene expression. After this time point, the amount of recombinant GFP stayed constant or slightly decreased. SDS-PAGE analysis clearly demonstrated the expected different relative molecular masses [M_r] of the various produced GFP fusion proteins which were in agreement with their calculated molecular masses (**Fig. 16A+B**). The shortest fusion protein was the untagged GFP with a calculated M_r of 27,800 (**Fig. 16A**, lane 2) while the protein with the highest M_r of 30,500 was Strep-TEV-GFP (**Fig. 16A**, lane 6). The molecular masses were calculated with the software Lasergene® (DNASTAR Inc.; Madison; USA).

Absolute protein amounts 6 h after induction of the *gfp* gene expression were deduced using a densitometric analysis of calibrated SDS-PAGE gels (**Fig. 16A**). Three distinct levels of GFP production were observed and compared with GFP amounts measured by luminescence spectrometer. The lowest production of GFP was observed for Strep-Xa-GFP (6.3 mg g_{CDW}^{-1}) and GFP-His (6.8 mg g_{CDW}^{-1}). An approximately 1.6-fold higher production was observed for GFP without tag (9.3 mg g_{CDW}^{-1}), Strep-TEV-GFP (10.5 mg g_{CDW}^{-1}) and GFP-Strep (11.2 mg g_{CDW}^{-1}). His-TEV-GFP (14.1 mg g_{CDW}^{-1}) was produced 2.2 times more than Strep-Xa-GFP and GFP-His. These differences between the 3 groups of GFP producers were observed in independent experiments with a standard deviation of less than 5 %. The comparison of fluorescence intensities of the various GFP producing cells (**Fig. 16C**) were in good agreement with the results of the densitometric analysis (**Fig. 16A**), with exception of Strep-Xa-GFP. Its relative fluorescence was found 1.4-fold reduced as also observed for the purified corresponding fusion protein. The relative fluorescence per mg GFP of all other fusion proteins was in a comparable range. Therefore, the correlation between fluorescence and amount of GFP-Strep (section III.1.2.2) could be further employed for determination the amount of all fusion forms of GFP via their fluorescence.

SDS-PAGE analysis of the soluble and insoluble fraction of intracellular proteins indicated that most of the recombinant GFP was found in the soluble fraction (> 90% of total GFP) (**Fig. 16A+B**). Only Strep-TEV-GFP (< 25 %) and His-TEV-GFP (< 30 %) were partly detected in the insoluble protein fraction (**Fig. 16B**, lanes 4+6).

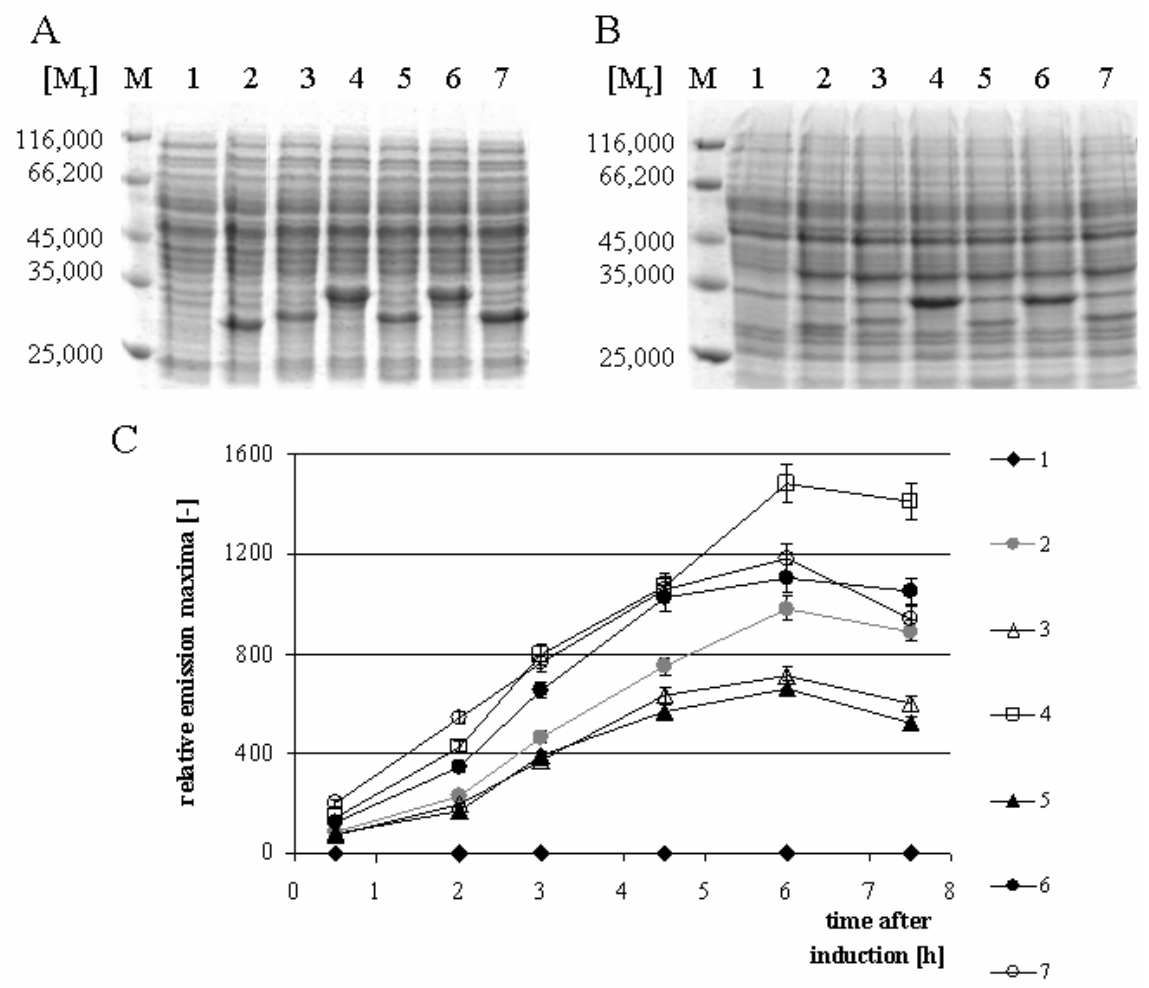


Figure 16: Production of different forms of affinity tagged GFP in *B. megaterium*. *B. megaterium* WH323 carrying the plasmids pSTOP1622 (target geneless plasmid, lane 1, ◆), pRBBm53 (encoding GFP, lane 2, ●), pRBBm35 (encoding GFP-His, lane 3, △), pRBBm55 (encoding His-TEV-GFP, lane 4, □), pRBBm36 (encoding Strep-Xa-GFP, lane 5, ▲), pRBBm54 (encoding Strep-TEV-GFP, lane 6, ●), and pRBBm56 (encoding GFP-Strep, lane 7, ○) were aerobically cultivated in 50 ml of LB medium at 30°C. The *gfp* gene expression was induced by the addition of 0.5 % (w/v) xylose at an OD_{578nm} of 0.4. **(A+B)** Six h after induction of the *gfp* gene expression, intracellular proteins of 0.5 x 10^9 of cells were separated in a soluble and an insoluble fraction and analysed via 15 % SDS-PAGE. The SDS-PAGE gel was stained with Coomassie Brilliant Blue G250. Lane M shows Protein Molecular Weight Marker (MBI Fermentas; St. Leon-Rot; Germany). **(A)** Soluble fractions. **(B)** Insoluble fractions. **(C)** Samples were taken 0.5, 2, 3, 4.5, 6, and 7.5 h after induction of the *gfp* gene expression. Cells were harvested by centrifugation, washed and diluted to a concentration of 10^9 cells per ml in 100 mM of sodium phosphate buffer (pH 7). The amount of GFP was determined via its fluorescence measured in a luminescence spectrometer (LS50B; PerkinElmer; Boston; USA). Samples were excited at 475 nm. Fluorescence emission maxima were detected at 512 nm.

The accessibility of the affinity tags fused to the N- and C-terminus of GFP was initially verified by Western blot analysis with antibodies directed against the His_6-tag and the StrepII-tag, respectively. Protein samples of 0.5 x 10^9 of cells 6 h after induction of the *gfp* gene expression were analysed. All tags were detectable in both soluble and insoluble protein fractions (**Fig. 17**) with different intensities compared to the SDS-PAGE. The anti-His antibody additionally detected a *B. megaterium* host protein (M_r of ~36,000, (**Fig. 17A1+2**)) which was observed in the Western blot analysis of proteins from *B. megaterium* carrying the target geneless plasmid pSTOP1622 as well (**Fig. 17**, lane 1). A natural His-tag was previously described for *B. megaterium* cobalt chelatase (CbiX, M_r = ~35,000) involved in vitamin B_{12} biosynthesis (Leech *et al.*, 2003). No production host related signals were detected using the streptavidin conjugate for detection of the StrepII-tag.

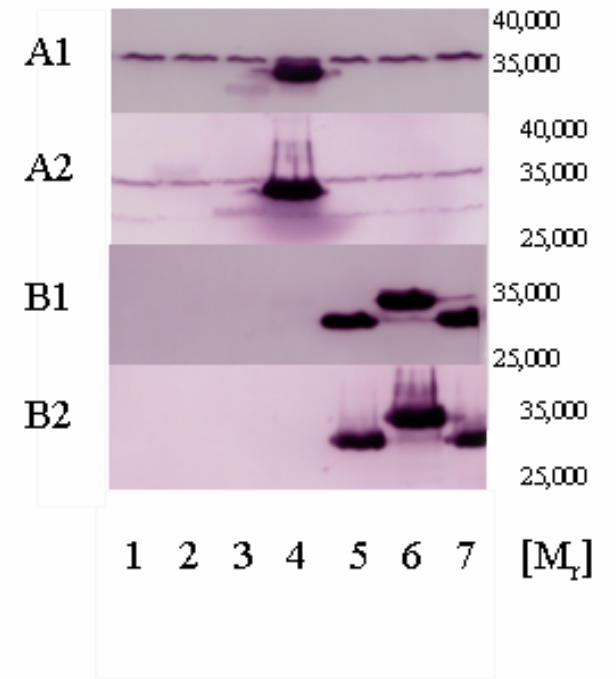


Figure 17: Western blot detection of GFP fusion proteins. Six h after induction of the *gfp* gene expression, intracellular proteins of 0.5 x 10^9 cells were separated by centrifugation in a soluble and an insoluble fraction and analysed via 15 % SDS-PAGE. The proteins were blotted onto PVDF membranes and the tags fused to GFP were detected using **(A)** the anti-His antibody or **(B)** the streptavidin conjugate for StrepII-tag detection. **(1)** Soluble fractions. **(2)** Insoluble fractions. Lane 1: negative control; lane 2: GFP; lane 3: GFP-His; lane 4: His-TEV-GFP; lane 5: Strep-Xa-GFP; lane 6: Strep-TEV-GFP; lane 7: GFP-Strep; lane M: PageRuler Prestained Protein Ladder (MBI Fermentas; St. Leon-Rot; Germany).

III.1.4 Purification of Affinity Tagged GFP from Cell-Free Extracts

Since the vector constructs proved to be applicable for expression, it was of further interest to purify the recombinant proteins. *B. megaterium* strains carrying the corresponding expression vectors encoding the various GFP fusion proteins were grown aerobically at 30°C in 50 ml of LB medium. After harvesting the cells 6 h after induction of the *gfp* gene expression, they were suspended in the corresponding binding buffer and disrupted via sonication. After ultra-centrifugation, the soluble GFP was visible by its green colour in the supernatant while almost no fluorescence was detected in the insoluble protein fractions.

Soluble GFP-His from 50 ml of cell culture was purified using a 200 µl Ni-NTA (nitrilotriacetic acid) column. Comparing fluorescence intensities of the protein solution before and after passage of the affinity column indicated that only 40 % of GFP-His had bound to the material. After several washing and pre-elution steps with 7 to 100 mM of imidazole, most of GFP-His (0.6 mg ml^{-1}) was eluted with 500 mM of imidazole (**Fig. 18A**, lane 1). Beside the main product, some additional bands indicated proteins with relative molecular masses of less than 8,000 up to 37,000. Considering only the main elution step, 2.4 mg of GFP per litre cell culture were purified which corresponded to 1.5 mg of purified GFP per g_{CDW}.

For His-TEV-GFP purification, the column volume was scaled up. To achieve an equal binding of His-TEV-GFP to the affinity material, a batch incubation for 1 h at 4°C of 1 ml of Ni-NTA sepharose with the cell-free extract from 50 ml of cell culture was preferred. Uniform binding of the fusion protein to the column observable through the green colour of the column was achieved. About 80 % of the His-TEV-GFP were bound to the sepharose. Washing with 7 to 100 mM of imidazole eluted most of the unspecifically bound proteins (data not shown). The elution with 200 mM of imidazole yielded 5.0 mg of His-TEV-GFP per litre cell culture (**Fig. 18A**, lane 2). However, the purified GFP showed two different sizes with relative molecular masses of 31,000 and 34,000. For both forms the same N-terminus was determined by N-terminal sequencing (R. Getzlaff; Helmholtz Centre for Infection Research; Braunschweig; Germany). Hence, a proteolytic degradation occurred near the C-terminus of the His-TEV-GFP. The N-terminal sequencing further demonstrated that for about half the amount of His-TEV-GFP the first methionine residue was removed.

Purified His-TEV-GFP showed its typical green fluorescence in SDS-PAGE if applied samples were not denatured by heating in SDS sample buffer prior to electrophoresis (Inouye and Tsuji, 1994). The non-denatured sample led to a modified protein pattern compared to the denatured forms. Two main fluorescent protein bands with a M_r of 32,000 and of 37,000 and a weak fluorescent band with a M_r of 30,000 were detected (**Fig. 18B**, lane 6). The observed degradation was insensitive to various protease inhibitor treatments.

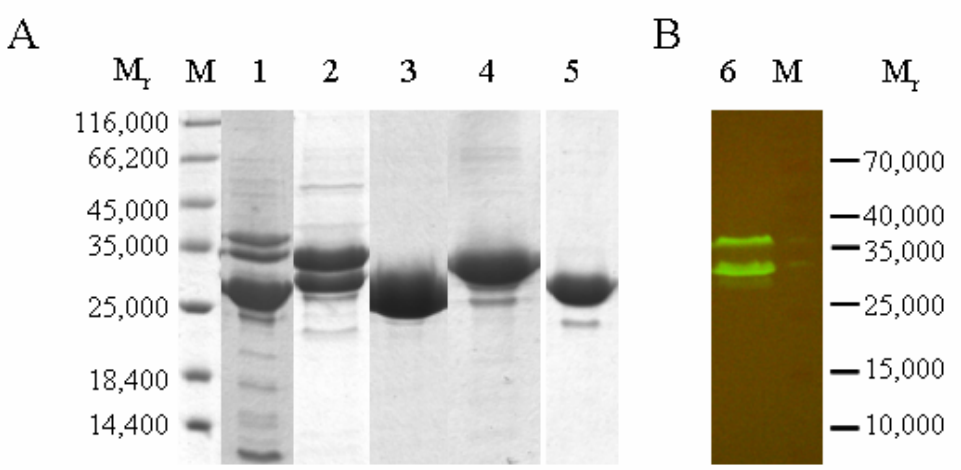


Figure 18: Purification of affinity tagged GFP recombinantly produced in different *B. megaterium* WH323 plasmid carrying strains. *B. megaterium* WH323 carrying the plasmids pRBBm35 (encoding GFP-His), pRBBm55 (encoding His-TEV-GFP), pRBBm36 (encoding Strep-Xa-GFP), pRBBm54 (encoding Strep-TEV-GFP), and pRBBm56 (encoding GFP-Strep) were aerobically cultivated in 50 ml of LB medium at 30°C. Cells were harvested 6 h after induction of the *gfp* gene expression and disrupted via sonication. Soluble proteins were obtained after centrifugation and loaded onto the corresponding affinity column. **(A)** Lane 1+2: Affinity chromatography using a Ni-NTA sepharose column. After loading and washing with 7 to 100 mM of imidazole, the proteins were eluted using 200 – 500 mM of imidazole. Lane 3-5: Affinity chromatography using Strep-Tactin sepharose. After loading and washing, StrepII-tagged GFP was eluted using 2.5 mM of desthiobiotin. **(A+B)** 20 µl of each fraction was analysed by SDS-PAGE after denaturation by heating. Only the major elution fractions are shown. Lane 1: GFP-His, lane 2: His-TEV-GFP, lane 3: Strep-Xa-GFP, lane 4: Strep-TEV-GFP, and lane 5: GFP-Strep, lane M: Protein Molecular Weight Marker (MBI Fermentas; St. Leon-Rot; Germany). **(B)** Fluorescence of 20 µl of purified His-TEV-GFP after separation via SDS-PAGE without prior heat denaturation (lane 6) was visualised under blue light using a yellow camera lens filter. Lane M: PageRuler Prestained Protein Ladder (MBI Fermentas; St. Leon-Rot; Germany).

Soluble GFP-Strep from 50 ml of cell culture was purified using an 1 ml Strep-Tactin affinity column; 10.8 mg of purified GFP-Strep per litre cell culture were obtained (**Fig. 18A**, lane 3). The purified GFP-Strep was used for the definition of the correlation between the amount of GFP and its corresponding fluorescence (section III.1.2.2). For purification of Strep-TEV-GFP by an 1 ml Strep-Tactin column, about 90 % of GFP were bound and 9 mg per litre of cell culture were purified. Beside the main protein band visualised on the SDS-PAGE, two weak bands corresponding to proteins with a M_r of approximately 27,000 and 29,000 were detected (**Fig. 18A**, lane 4). Again, these proteins were identified as degradation forms of GFP based on N-terminal protease digestion which could not be eliminated by adding protease inhibitors. After purification of Strep-Xa-GFP using the same operation around 6 mg per litre of cell culture were received (**Fig. 18A**, lane 5). Comparison of 9 mg of Strep-TEV-

GFP and 6 mg Strep-Xa-GFP represented the ratio of these GFP-forms in the cells (section III.1.3) with a factor of 1.5. The amounts of purified GFP are summarised in **table 9**.

Table 9: Comparison of production and purification of GFP. Different affinity tag fusion forms of GFP were produced in *B. megaterium* WH323. Purification was performed using affinity chromatography. Amounts of purified GFP-Strep were determined using a Bradford protein assay kit (Bio-Rad; Munich; Germany) and BSA (Perbio; Rockford; USA) as standard. Amounts of purified GFP-His, His-TEV-GFP, Strep-Xa-GFP, and Strep-TEV-GFP were calculated via their relative fluorescence per mg protein.

	Protein produced		Protein purified	
Protein	[mg l^{-1} cell culture]	[mg g_{CDW}^{-1}]	[mg l^{-1} cell culture]	[mg g_{CDW}^{-1}]
GFP-His	9.6 +/- 0.5	6.8 +/- 0.3	2.4 +/- 0.1	1.5 +/- 0.1
His-TEV-GFP	17.9 +/- 0.9	14.0 +/- 0.7	5.0 +/- 0.3	3.0 +/- 0.2
Strep-Xa-GFP	8.4 +/- 0.4	6.3 +/- 0.3	6.0 +/- 0.3	4.0 +/- 0.2
Strep-TEV-GFP	13.2 +/- 0.7	10.5 +/- 0.5	9.0 +/- 0.5	6.0 +/- 0.3
GFP-Strep	16.0 +/- 0.8	11.2 +/- 0.6	10.8 +/- 0.5	6.9 +/- 0.4

III.1.4.1 Removal of Affinity Tags of Purified GFP Fusion Proteins

After purification of affinity tagged GFP fusion proteins possessing a protease cleavage site for Factor Xa or TEV protease, the N-terminally fused affinity tags were eliminated. For 1 mg of Strep-Xa-GFP the addition of 64 µg of Factor Xa achieved the best elimination of the StrepII-tag overnight at 23°C. The minor band with a M_r of approximately 32,000 represents Factor Xa protease (**Fig. 19A**, lane b). The incubation of 6.3 µg of fusion protein without Factor Xa showed that Strep-Xa-GFP was *per se* stable (**Fig. 19A**, lane a). Using the TEV protease, the N-terminal StrepII-tag as well as the N-terminal His_6-tag were successfully cleaved. Best results were obtained using 80 µg of TEV protease per mg fusion protein. Complete cleavage was achieved overnight at 20°C (**Fig. 19A**, lane d). After incubation, the TEV protease was detectable with a relative molecular mass of approximately 28,000 in the SDS-PAGE analysis (**Fig. 19A**, lane d). Incubation of 5.8 µg of purified Strep-TEV-GFP under identical conditions in the absence of TEV protease at 20°C did not harm the recombinant fusion protein (**Fig. 19A**, lane c).

After successful cleavage of both employed affinity tags using Factor Xa or TEV protease, removal of the protease was desired. For this purpose, 200 µg of concentrated His-TEV-GFP were separated into two portions. One half was incubated at 20°C with and the other without addition of His_6-tagged TEV protease. After incubation, both portions were separately applied

to Ni-NTA affinity chromatography. As expected, GFP still fused to its His_6-tag, the cleaved-off His_6-tag and the His_6-tagged TEV protease were all found bound to the affinity material and were only released from the column material by the addition of imidazole. In contrast, processed GFP without His_6-tag did not bind. Accordingly, the His-TEV-GFP without TEV protease treatment was exclusively found in the imidazole elution steps (**Fig. 19BI**, E2-4). After incubation with the TEV protease, GFP was mainly found in the flow-through indicating the successful cleavage of the His_6-tag of the GFP (**Fig. 19BII**, FT).

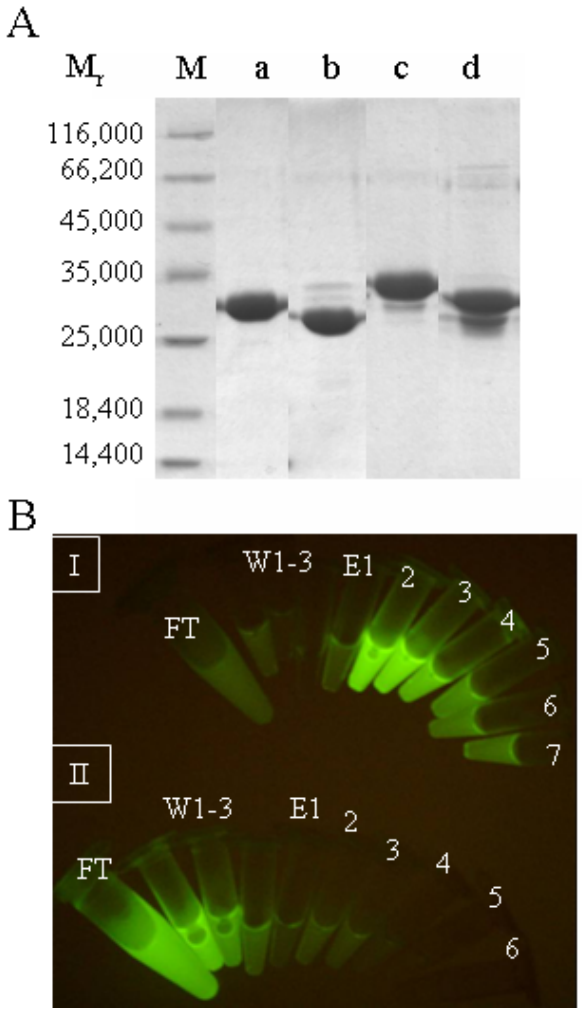


Figure 19: Removal of the affinity tags from the GFP fusion proteins. After purification of Strep-Xa-GFP, Strep-TEV-GFP and His-TEV-GFP (for details see **Fig. 18**), the affinity tags were removed. **(A)** Protease cleavage of Strep-Xa-GFP and Strep-TEV-GFP. 1 mg of purified Strep-Xa-GFP and Strep-TEV-GFP were incubated with 64 µg of Factor Xa and 80 µg of TEV protease at 23°C and 20°C overnight, respectively. 6.3 µg of Strep-Xa-GFP before (lane a) and after (lane b) Factor Xa protease addition and 5.8 µg of Strep-TEV-GFP before (lane c) and after (lane d) TEV protease addition were separated via SDS-PAGE. Lane M: Protein Molecular Weight Marker (MBI Fermentas; St. Leon-Rot; Germany). The SDS-PAGE gel was stained by Coomassie Brilliant Blue G250. **(B)** After purification of His-TEV-GFP using a Ni-NTA affinity chromatography column, 100 µg of His-TEV-GFP were incubated with or without His_6-tagged TEV protease overnight at 20°C. In each case, the total volume was loaded onto a Ni-NTA affinity column. **(BI)** Without protease addition, the His-TEV-GFP bound to the affinity material. After washing (W1-3), the elution with 200 mM of imidazole released the His-TEV-GFP from the affinity material (E1-7). **(BII)** After addition of TEV protease, GFP without His_6-tag did not bind to the Ni-NTA affinity column. It was found in the flow-through (FT) and in the washing fractions (W1-3). The elution fractions showed less (E1-3) or no (E4-6) fluorescence. GFP was visualised on a light table emitting blue light and a yellow filter in front of the camera lens.

III.1.5 Thioredoxin TrxA from *Bacillus megaterium* as Fusion Partner for GFP

The electron transferring thioredoxin, a protein with a M_r of 12,000, is known from plants, yeast and bacteria. It was first isolated from *E. coli* as electron donor for the ribonucleotid reductase (Laurent *et al.*, 1964). The linkage of thioredoxin to target proteins was described to increase the solubility of the resulting fusion proteins in the cytoplasm of *E. coli* and thereby prevents the formation of inclusion bodies (LaVallie *et al.*, 1993; Stewart *et al.*, 1998).

GFP produced in *B. megaterium* resulted in up to 30 % of insoluble protein using the constructs pRBBm54 (encoding Strep-TEV-GFP) and pRBBm55 (encoding His-TEV-GFP). We wanted to test if an N-terminal fusion to thioredoxin leads to an increase of soluble GFP. However, the production of *E. coli* thioredoxin (TrxA) in *B. megaterium* was not successful (Drews, 2004; Stammen, 2005). One reason could be the low CAI-value of *E. coli trxA* with only 0.35 for *B. megaterium*. *B. megaterium* TrxA, consisting of 104 aa, shows 50 % identity to *E. coli* TrxA. The *B. megaterium trxA* gene was amplified and cloned in frame upstream of the coding sequence *strep-TEV-gfp* of pRBBm54 and *his-TEV-gfp* of pRBBm55 resulting in pRBBm67 and pRBBm68, respectively. The introduced TEV protease cleavage site is useful to remove TrxA after production and purification.

After protoplast transformation of *B. megaterium* WH323 with these novel constructed plasmids, WH323 carrying pRBBm54 (encoding Strep-TEV-GFP), pRBBm55 (encoding His-TEV-GFP), pRBBm67 (encoding TrxA-Strep-TEV-GFP), and pRBBm68 (encoding TrxA-His-TEV-GFP), respectively, were grown aerobically in LB medium at 30°C. Recombinant gene expression was induced by the addition of 0.5 % (w/v) xylose at an OD_{578nm} of 0.4. Growth was followed by the measurements of the OD_{578nm}. Samples for protein analysis via SDS-PAGE and fluorescence spectroscopy were taken 0.5, 2, 3, 4.5, 6, 7.5, and 9 h after induction of recombinant gene expression. All strains showed comparable growth profiles reaching the stationary phase between 6 and 7.5 h after induction of the *gfp* gene expression with a final OD_{578nm} of about 4.3. Comparing the maximal production of Strep-TEV-GFP (13.9 mg l^{-1}) and His-TEV-GFP (17.5 mg l^{-1}) with that of the TrxA-fusion proteins indicated that 5.5 times less of TrxA-Strep-TEV-GFP (2.5 mg l^{-1}) and 7.6 times less of TrxA-His-TEV-GFP (2.3 mg l^{-1}) were produced. In SDS-PAGE analysis neither in the soluble nor in the insoluble proteins fractions corresponding protein bands for the TrxA-fusions were detected (data not shown). Hence, the N-terminal fusion of GFP to TrxA led to a drastic decrease of the total GFP production.

III.1.6 Employment of New Origins of Replication for *Bacillus megaterium* Vector Systems

In *E. coli* two parallel replicating vectors are commonly used. There are different reasons for the application of so called two vector systems in this organism. One is the co-production of vector encoded additional tRNAs for rare codons resulted in so called "codon plus" strains (summarised in Makrides, 1996). Furthermore, *E. coli* pLysS strains, constructed for recombinant gene expression, are carrying a plasmid encoding the T7 lysozyme, a natural inhibitor of T7 RNA polymerase, which suppresses the basal expression from the T7-promoter (Stano and Patel, 2004). Furthermore, the initial T7-promoter system was based on a two vector system (Tabor and Richardson, 1985).

For stable in parallel replication of two vectors, two different origins of replication (*ori*) and selection markers are required. Here, a novel two vector system for *B. megaterium* was tested with recombinantly produced GFP as model protein.

The origin of replication *oriU* and the gene *repU* encoded on pMM1520 (Malten *et al.*, 2005a) and all derivatives of this plasmids are derived from the plasmid pBC16 (Bernhard *et al.*, 1978) which is replicated by the rolling circle mechanism (Khan, 2005). Two further origins of replication, *repM100* and *repM700* were used in this study. They are derived from plasmid pBM100 (Kieselburg *et al.*, 1984) and pBM700 (Stevenson *et al.*, 1998), respectively, which were isolated from *B. megaterium* strain QM B1551 (Kieselburg *et al.*, 1984). Replication origin *repM100* necessary for plasmid replication of pMGBm19 (Gamer, 2006; personal gift) is derived from the plasmid pYZ5 which is a truncated form of pYZ11 (Kunnimalaiyaan *et al.*, 2001). These plasmids as well replicate by the rolling circle mechanism. For pMGBm19, the xylose-inducible promoter system and the MCS of pMM1520 were inserted into pYZ5. Plasmid pMGBm21 (Gamer, 2006; personal gift) was constructed by inserting the xylose-inducible promoter system and the MCS of pMM1520 into pKM704 (Vary, personal gift). Latter is a vector which is basically derived from pBM700. Here, plasmid replication is mediated by the theta plasmid replicon *repM700*. The replicions *repU*, *repM100* and *repM700* belong to different compatibility classes. Hence, pMM1520, pMGBm19 and pMGBm21 and their derivatives can be replicated in parallel by *B. megaterium*. The selection marker encoded on pMM1520 mediates tetracycline resistance while the gene responsible for chloramphenicol resistance is encoded by pMGBm19 and pMGBm21.

III.1.6.1 GFP Production Using Free Replicating Plasmids Based on Different Origins of Replication

The *gfp* gene was amplified by PCR and inserted into pMM1522, pMGBm19, and pMGBm21 resulting in pRBBm34, pRBBm63, and pRBBm64, respectively. After protoplast transformation of *B. megaterium* WH323 with these plasmids, cells were grown aerobically in LB medium supplemented with the corresponding antibiotic at 30°C. The *gfp* gene expression was induced by the addition of 0.5 % (w/v) xylose at an OD_{578nm} of 0.4. Samples for measurements of the OD_{578nm} and for GFP analysis via SDS-PAGE and fluorescence spectroscopy were taken 0.5, 2, 3, 4.5, 6, 7.5, and 9 h after induction of the *gfp* gene expression.

All strains reached the stationary phase 6 h after induction of the *gfp* gene expression. WH323 carrying the theta-replicating plasmid pRBBm64 reached highest cell densities with a final OD_{578nm} of 6.0 where as WH323 carrying pRBBm34 or pRBBm63 showed final maximal OD_{578nm} of 5.0. In all plasmid carrying strains, highest intracellular GFP amounts were measured 6 h after induction of the *gfp* gene expression. Only 3.5 mg GFP per litre growth medium were produced by WH323 carrying pRBBm64 which was about 4 times less compared to GFP production mediated by the rolling circle plasmids pRBBm34 and pRBBm63. As expected, WH323 carrying pRBBm34 (11.9 +/- 0.6 mg GFP l^{-1}) or pRBBm63 (13.8 +/- 0.7 mg GFP l^{-1}) showed only little differences in GFP-production.

Therefore, derivatives of the rolling circle plasmids pMM1520 and pMGBm19 were used for further experiments. Plasmid pMGBm21 replicating by a theta replicon mechanism may be used for xylose controlled target gene expression if the target gene product is required in little amounts. For increasing recombinant protein production it does not seem suitable.

III.1.6.2 GFP Production Using a Two Vector System

In order to investigate how a two vector system does effect the production of GFP, strains carrying either one GFP encoding and one target geneless plasmid or two GFP encoding plasmids were used. To identify the plasmid responsible for GFP production, *B. megaterium* plasmid strain WH323 carrying pRBBm54 (encoding Strep-TEV-GFP) instead of WH323 carrying pRBBm34 (encoding GFP) were chosen for further studies because GFP and Strep-TEV-GFP were distinguishable in SDS-PAGE analysis by their sizes (**Fig. 16A**, lane 2+6). First, *B. megaterium* WH323 carrying pRBBm54 (encoding Strep-TEV-GFP) was protoplasted and transformed with pRBBm63 (encoding GFP) and the target geneless

pMGBm19, respectively. In a second experiment, WH323 carrying pRBBm63 (encoding GFP) was transformed with the target geneless pSTOP1622 (**Tab. 10**). Expression studies occurred in LB medium supplemented with 10 µg ml^{-1} of tetracycline and 4.5 µg ml^{-1} of chloramphenicol. The *gfp* gene expression was induced by the addition of 1 % (w/v) xylose at an OD_{578nm} of 0.4. Growth was followed by measurements of the OD_{578nm}. Samples for GFP analysis via SDS-PAGE and fluorescence spectroscopy were taken 0.5, 2, 3, 4.5, 6, 7.5, and 9 h after induction of the *gfp* gene expression. Obviously, the growth of *B. megaterium* strains carrying two plasmids in LB medium supplemented with two antibiotics was less efficient compared to strains carrying only one plasmid. The stationary phase was reached 7.5 h after induction of the *gfp* gene expression with a final OD_{578nm} of less then 3.5. SDS-PAGE analysis of the intracellular proteins indicated that GFP as well as Strep-TEV-GFP were produced in same amounts in WH323 carrying pRBBm54 and pRBBm63 (data not shown). Maximal GFP production was detected 7.5 h after induction of the *gfp* gene expression. The results are summarised in **table 10**.

Table 10: Comparison of GFP production 7.5 h after induction of the *gfp* gene expression in *B. megaterium* strain WH323 carrying one or two plasmids. WH323 plasmid carrying strains were grown aerobically in 50 ml of LB shaking flask cultures at 30°C. The *gfp* gene expression was induced at OD_{578nm} of 0.4 with 1.0 % (w/v) xylose. 7.5 h after induction of recombinant gene expression, amounts of intracellular produced GFP were measured spectroscopically.

B. megaterium WH323 carrying		GFP produced	
plasmid I (tetracycline resistance)	plasmid II (chloramphenicol resistance)	[mg l^{-1} cell culture]	[mg g_{CDW}^{-1}]
-	pRBBm63 (encoding GFP)	13.9 +/- 0.7	7.8 +/- 0.4
pRBBm54 (encoding Strep-TEV-GFP)	-	13.2 +/- 0.7	10.5 +/- 0.5
pSTOP1622 (target geneless)	pRBBm63 (encoding GFP)	7.1 +/- 0.4	5.9 +/- 0.3
pRBBm54 (encoding Strep-TEV-GFP)	pMGBm19 (target geneless)	5.6 +/- 0.3	4.8 +/- 0.2
pRBBm54 (encoding Strep-TEV-GFP)	pRBBm63 (encoding GFP)	16.4 +/- 0.8	14.6 +/- 0.7

Although WH323 carrying pRBBm63 (13.9 +/- 0.7 mg GFP l^{-1}) and WH323 carrying pRBBm54 (13.2 +/- 0.7 mg Strep-TEV-GFP l^{-1}) produced the same amount of GFP (**Tab. 10**), the presence of a second target geneless vector resulted in significant differences. The amount of GFP was reduced to about 50 % (7.1 mg GFP l^{-1}) and 58 % (5.6 mg Strep-TEV-GFP l^{-1}) while the specific amount was still 75 % (5.9 mg GFP g_{CDW}^{-1}) and 45 % (4.8 mg Strep-TEV-GFP g_{CDW}^{-1}) compared to the strains carrying only one plasmid mediating GFP-production. Furthermore, 1.3 times more GFP (7.1 +/- 0.4 mg l^{-1}) than Strep-TEV-GFP (5.6 +/- 0.3 mg l^{-1}) was produced. The plasmids encoded production of two GFP forms and additionally of the chloramphenicol acetyl transferase and the tetracycline resistance protein as well as the DNA replication of two plasmids seemed to constitute an increased metabolic burden for the host bacterium (Glick, 1995). This may roughly explain the decrease of GFP production.

As expected, a second GFP encoding plasmid resulted in an enhancement of its intracellular amount compared to the GFP-production mediated by only one plasmid. The volumetric GFP amount increased 1.2 times to 16.4 mg l^{-1} while the specific amount arose up to 1.9 times to 14.6 mg g_{CDW}^{-1}. The influence of the metabolic burden on protein production was already mentioned above. Compared to the GFP-production in the *B. megaterium* plasmid strains carrying two plasmids – one encoding GFP and one target geneless – the increase was higher. The volumetric GFP amount was enhanced 2.3 and 2.9 times, respectively, while the specific amount increased 2.4 and 3 times, respectively.

Hence, it was shown that the in parallel replication of two vectors and the GFP production mediated by two vectors is possible. Nevertheless, due to the higher metabolic burden, the use of a second vector did not consequently result in a proportional increase of recombinant produced GFP.

Anyway, this novel vector system might be useful for time-delayed coexpression of different genes under the control of different inducible promoters (section III.3). Moreover, two in parallel replicating vectors with different copy numbers may provide the basic for a T7 RNA polymerase-dependent promoter system. The T7 RNA polymerase under control of the xylose-inducible promoter may be encoded by one vector while the target gene under control of the T7 promoter may be located on the second one.

III.1.6.3 Influence of Antibiotics on *Bacillus megaterium* Growth Profiles and GFP Production

B. megaterium is known for the stable maintenance of plasmids over many generations without the addition of any selection marker (Rygus *et al.*, 1991; Vary, 1992; von Tersch and Robbins, 1990). Here, the influence of recombinant gene expression on the plasmid segregation was tested.

Therefore, a pre-culture of *B. megaterium* WH323 carrying the two plasmids pRBBm54 and pRBBm63 was aerobically cultivated in LB medium without the addition of any antibiotic. The main cultivation, however, was performed in LB medium with or without the addition of the corresponding antibiotics at 30°C. The *gfp* gene expression was induced at an OD_{578nm} of 0.4 by the addition of 1 % (w/v) xylose. 0.5, 2, 3, 4.5, 6, 7.5, and 9 h after induction of the *gfp* gene expression, samples for measurements of the OD_{578nm} and for GFP analysis via SDS-PAGE and spectroscopy were taken. The growth profile of the main culture shifted to LB medium supplemented with antibiotics showed, as expected, a longer lag phase. The stationary phase was reached 7.5 h after induction of the *gfp* gene expression with a final OD_{578nm} of less then 3.0. The maximal amount of recombinant GFP achieved 9 h after induction of the *gfp* gene expression was equal to that of a not shifted culture (**Tab. 10**) with 14.3 mg l^{-1} and 14.9 mg g_{CDW}^{-1}, respectively. Lacking antibiotic addition to the main culture resulted in a faster growth with higher cell densities. The stationary phase was reached 6 h after induction of the *gfp* gene expression with a final OD_{578nm} of 4.2. Anyway, the production of GFP was reduced up to 82 % with 4 mg l^{-1} and 2.7 mg g_{CDW}^{-1}, respectively. These intracellular GFP amounts were achieved 3 to 4.5 h after induction of the recombinant *gfp* gene expression and remained constant for the next hours.

These results support the idea of maintenance of plasmids in *B. megaterium* with selection marker during protein production.

III.1.7 High Cell Density Cultivations (HCDCs)

III.1.7.1 HCDC of *Bacillus megaterium* WH323 Carrying pRBBm56 (GFP-Strep)

III.1.7.1.1 HCDC of *Bacillus megaterium* with Early Induction of Recombinant Gene Expression

After demonstrating the feasibility of GFP production at small scale, the process was transferred into a bioreactor to study GFP production in a glucose limited fed-batch cultivation where high cell densities are reached (Hollmann and Deckwer, 2004; Malten *et al.*, 2005a). *B. megaterium* strain WH323 carrying pRBBm56 (encoding GFP-Strep) was chosen, since it produced high amounts of GFP-Strep fusion protein obtainable as an apparently pure protein after a one-step purification process (**Fig. 18A**, lane 3). A5 medium with 30 g l^{-1} of glucose was used for an 1 litre batch cultivation (**Fig. 20AI**) (Malten *et al.*, 2005a). About 2 h prior to the end of the batch phase (11.2 h) recombinant gene expression was induced by the addition of 0.5 % (w/v) xylose leading to the production of GFP-Strep which was measured via its fluorescence (**Fig. 20AI**). After exhaustion of the glucose (13.3 h), further glucose was fed exponentially into the growing culture theoretically setting the growth rate (μ_{set}) to 0.12 h^{-1}. The feeding formula (Malten *et al.*, 2005a) did not account for the maintenance metabolism. Hence, an actual μ_{set} of 0.08 h^{-1} was obtained. During this initial stage, the amount of GFP increased from zero up to 11.2 mg of GFP per g_{CDW} and remained constant for the next 9 h. In the meantime, the biomass concentration further increased in parallel to the cell density resulting in a maximum of the volumetric GFP concentration of 226 mg of GFP per litre at 25 h and 20 g CDW l^{-1}. Hence, at this time point the cultivation should be terminated for maximal protein yields. When specific GFP production rates were calculated, it was observed that the highest specific production rates were reached at about 3.5 mg of GFP per g_{CDW} and h directly after xylose addition (11.2 h). In contrast, volumetric production rates stayed constant at a level of approximately 24 mg of GFP per litre and h for 10 h after induction.

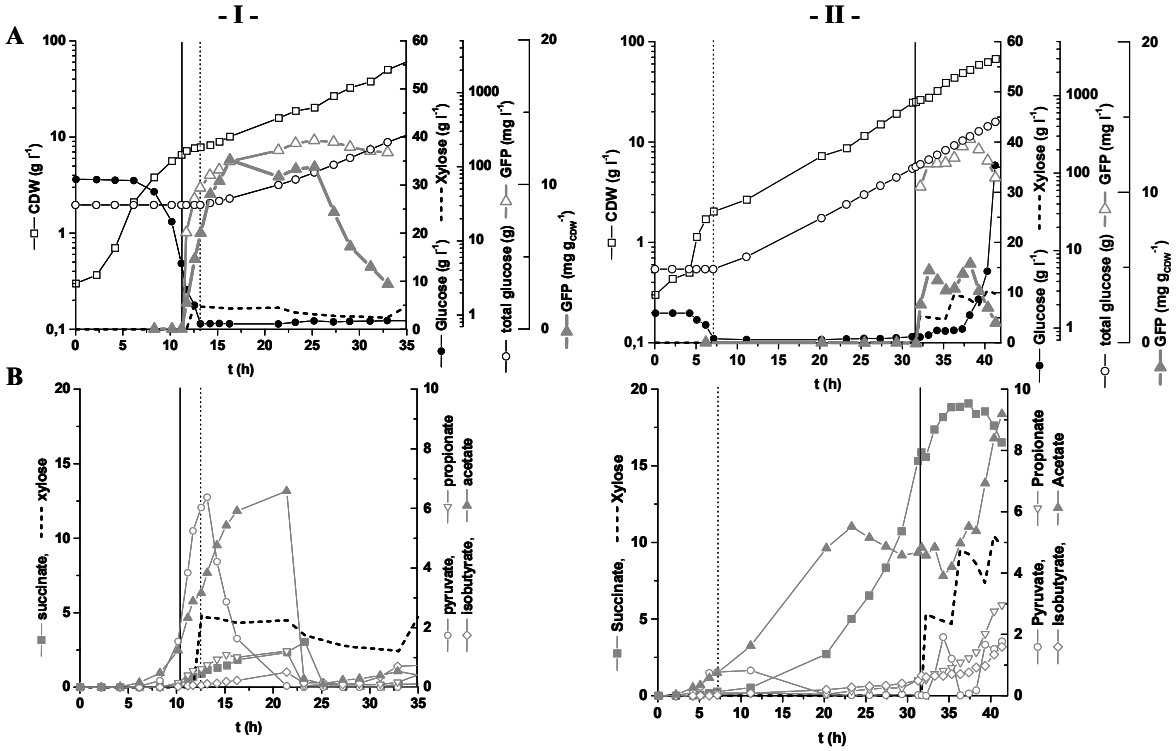


Figure 20: High cell density cultivation of *B. megaterium* WH323 carrying pRBBm56 encoding GFP-Strep. The *gfp* gene expression was induced by the addition of 0.5 % (w/v) xylose in a first run at 6.5 g CDW l^{-1} **(-I-)** and in a second run at 25 g CDW l^{-1} **(-II-)**. Growth and GFP production profiles **(A)** and metabolites detected by HPLC [g l^{-1}] **(B)** are shown. The solid line illustrates induction of the *gfp* gene expression. The start of the exponential glucose feeding profile is indicated by a dashed line.

III.1.7.1.2 HCDC of *Bacillus megaterium* with Late Induction of Recombinant Gene Expression

In a second fed-batch cultivation, a different induction profile was tested (**Fig. 20II**). In order to obtain a better yield, GFP production was induced at a higher biomass concentration. Here, the batch cultivation was started with 4 g of glucose per litre trying to avoid the formation of organic acids in the batch phase as seen in the case of early induction (**Fig. 20BI**). The exponential feeding was started directly after the exhaustion of glucose with μ_{set} of 0.14 h^{-1} resulting in an actual μ_{set} of 0.11 h^{-1}. Five g of xylose per litre were added at 25 g CDW l^{-1} (31.7 h, **Fig. 20AII**) for induction of the *gfp* gene expression. The onset of GFP production quickly led to 4.8 mg of GFP per g_{CDW}. However, intracellular GFP amounts decreased in the next 2 h to 3.4 mg of GFP per g_{CDW}. Due to the decrease and the low yield of GFP per cell compared to the previous fed-batch experiment, a second addition of 5 g l^{-1} xylose was attempted. This second xylose addition increased the cellular GFP amount to 5.2 mg of GFP per g_{CDW} and also led to a maximal volumetric GFP amount of 274 mg of GFP l^{-1} at a

biomass concentration of 52 g CDW l^{-1} (38.2 h). Further on, specific and volumetric GFP amounts decreased while glucose concentrations increased. A third xylose addition (39 h) showed no positive effect on the amount of recombinant GFP. The calculation of specific GFP production rates demonstrated its rapid increase after the first and second induction to maximal values of 4.7 and 1.0 mg of GFP per g_{CDW} and h, respectively.

III.1.7.1.3 Comparison of HCDC with Early and Late Induction of Recombinant Gene Expression

A decrease of the inducer xylose in the growth medium was observed in both fed-batch cultivations (**Fig. 20A**). A mass balance of the bioreactor demonstrated that the total amount of xylose was constant. Hence, xylose was not consumed by *B. megaterium* and the decrease in xylose concentration was only caused by the addition of new feed and base solution.

In both fed-batch cultivations organic acids, especially pyruvate, acetate and succinate, were not only formed in the batch phase, but also during glucose limited feeding. The higher initial glucose concentration in the first cultivation compared to the second led to a higher concentration of organic acids in the batch phase (**Fig. 20B**).

In both experiments pyruvate formed during the batch phase was consumed in the fed-batch phase. Mainly acetate was further accumulated during the fed-batch phase in the first experiment. Formed organic acids were metabolised successively during glucose limitation. After the exhaustion of pyruvate (22 h), the concentration of acetate followed by that of succinate decreased. With the late induction of the *gfp* gene expression, acetate was also formed and diminished slightly again after exhaustion of pyruvate. Due to the at this point still high acetate levels, succinate was further accumulated to a maximal concentration of 19 g l^{-1}.

This fed-batch cultivation demonstrated that the early and the late induction of the *gfp* gene expression are feasible strategies for high cell density protein production. The xylose-dependent promoter was tightly controlled independently of cell density. In contrast, in *E. coli* the *gfp* gene expression under T7/lac-promoter control was leaky especially at the here used low growth rates of 0.1 h^{-1} (Reischer *et al.*, 2004). Moreover, the lowest obtained specific GFP amount of 5.2 mg of GFP per g_{CDW} in *B. megaterium* was still 1.6-fold higher than in an *E. coli* chemostat culture at a comparable growth rate of 0.1 h^{-1}, where 3.3 mg GFP per g_{CDW} were measured (Reischer *et al.*, 2004).

Constancy of the specific GFP production rate in *B. megaterium* was only obtained if GFP production was started early (**Fig. 20I**). In the late induced fed-batch cultivation, specific GFP

amounts decreased quickly. This might be due to either high succinate concentrations during GFP production or the dilution derived decrease in xylose concentration. It is known that *Bacillus* fed-batch cultivations are prone to acidification caused by an overflow metabolism from glucose (Yoon *et al.*, 1994). Here, glucose was only present in excess in the batch phase. However, major accumulation of organic acids also occurred during glucose limiting fed-batch. A limitation of trace elements was observed and corrected by discontinuously trace element addition when carbon dioxide formation decreased. This may have led to a temporary excess of glucose causing an onset of organic acid production. Control of organic acid formation and consumption seems to be complex in *B. megaterium* as exemplified by the organic acid concentration profiles.

III.1.7.2 Fluorescent Activated Cell Sorting (FACS) Analysis of GFP-Production in *Bacillus megaterium* Cells

To study the effect of GFP production on host viability and the productivity of single *B. megaterium* cells, the fluorescence of the model protein GFP was combined with a live/dead stain using propidium iodide (PI) in a flow cytometric analysis. *B. megaterium* cells had a slight but significant red and green auto-fluorescence visible on a green (FL1) and red (FL3) fluorescence dot plot in a flow cytometric analysis without additional staining. For separation of alive and dead cells, samples were stained with PI, a hydrophobic fluorescent molecule that exclusively passes damaged cell membranes. PI intensively stained dead cells in a control (**Fig. 21C**), whereas living cells showed only minimal fluorescence (**Fig. 21A**). Furthermore, induction of the *gfp* gene expression by xylose addition led to an increase in the intensity of green fluorescence of living cells (**Fig. 21B**) and of dead cells which have started to produce GFP during live time (**Fig. 21D**). Hence, a quadrant gating on the red-green fluorescence dot blot separated the investigated cells into four subpopulations (**Fig. 21F**): 1. living cells producing GFP (green), 2. living cells not producing GFP (red), 3. dead cells containing GFP (cyan), 4. dead cells not containing GFP (black). An analysis of samples from the fed-batch cultivation with induction at 25 g CDW l^{-1} revealed that no GFP was produced prior to induction of the *gfp* gene expression controlled by the xylose-inducible promoter P_{xylA}. 30 min after induction, 76 % of all cells produced active GFP. Within the next 4 h only 3 % of the GFP producing cells died. Hence, recombinant GFP production only had a minor effect on the viability of the cell population (**Fig. 21G**).

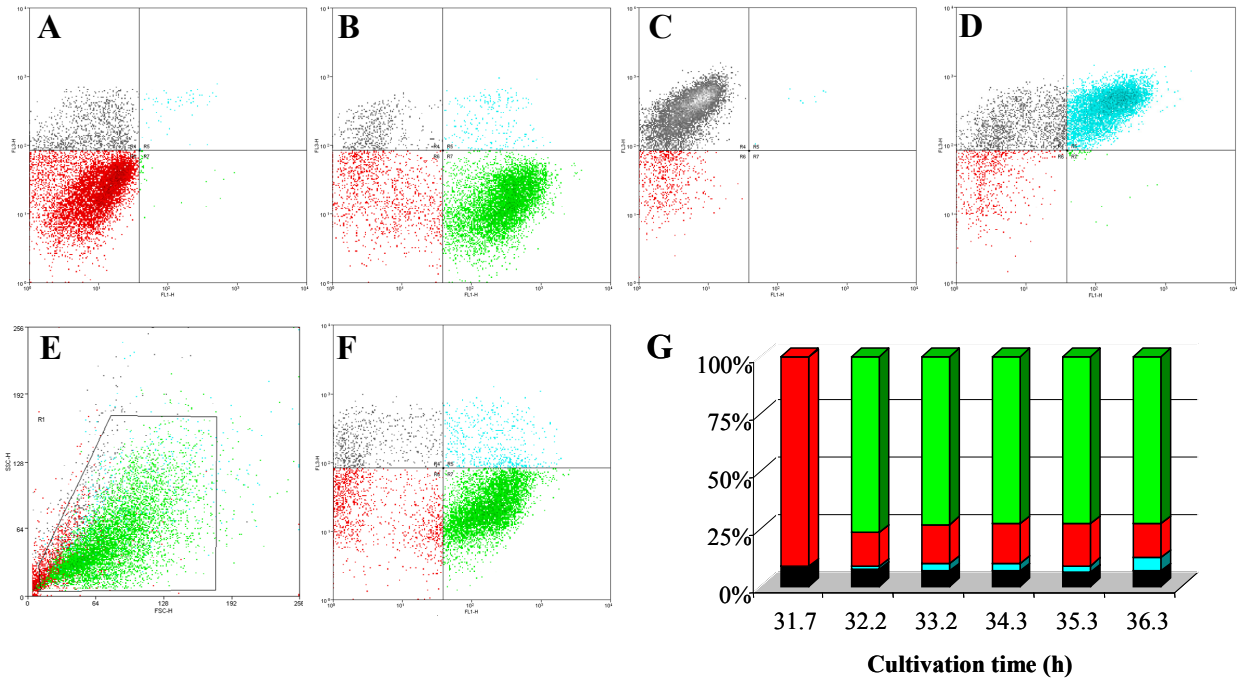


Figure 21: Flow cytometric analysis and GFP production of cells in an HCDC fermentation. Samples taken from the fed-batch of *B. megaterium* WH323 carrying pRBBm56 (GFP-Strep) before and after induction of the *gfp* gene expression at 25 g CDW l^{-1} were stained with propidium iodide (PI) and analysed in a FACSCalibur (Benton Dickinson; Erembodegen-Aalst; Belgium) for red (FL3) and green (FL1) fluorescence **(A-D, F)**. As controls, fed-batch samples of living (**A**, red), living and GFP producing (**B**, green), dead (**C**, black) and dead and GFP producing (**D**, cyan) cells are shown. **(E)** The gate, used for its FL3 / FL1 dot blot. **(F)** Side (SSC) and frontal (FSC) scatter of a sample taken at 36.3 h are indicated by a box. **(G)** Accordingly coloured columns show percentage of the subpopulation compared to all cells.

III.2 EXTRACELLULAR PRODUCTION OF RECOMBINANT PROTEINS

After the successful development of vectors for the intracellular production and purification of recombinant proteins using *B. megaterium*, this system was expanded with vectors for the extracellular recombinant protein production and the following affinity tag aided protein purification from the cell-free growth medium.

III.2.1 Construction of the Protein Secretion Vector pMM1525

For the secretion of heterologous proteins in a *B. megaterium* host, a vector encoding a functional signal peptide was constructed. This vector allows a translational fusion of the gene of interest to the signal peptide encoding sequence. The resulting fusion protein is destined for the transport via the SEC-pathway into the growth medium. In order to achieve efficient secretion and processing, a homologous *B. megaterium* signal peptide was chosen for the secretion of recombinant proteins. The recently discovered extracellular *B. megaterium* esterase LipA (Ruiz *et al.*, 2002) was found secreted in high amounts into the culture medium of *B. megaterium*. The LipA signal peptide contains 28 amino acids. The three typical regions of a SEC-pathway dependent signal peptide were identified in the LipA signal peptide by the SignalP algorithm (Nielsen and Krogh, 1998). Its cleavage site for the signal peptidase type I consists of an AKA motif (**Fig. 22A)**. The second amino acid of the type I signal peptidase recognition site is variable (Tjalsma *et al.*, 2000). Therefore, it was possible to incorporate the *Sfo*I restriction site in the coding sequence by substituting the lysine residue (K) by a glycine residue (G). A glycine residue at this position was found in 6 % of the secreted proteins in *B. subtilis* in comparison to a 12 % frequency for the lysine residue (Antelmann *et al.*, 2001). The introduced *Sfo*I restriction site allows the cloning of genes directly adjacent to the signal peptide coding sequence (**Fig. 22B**). As a consequence, the resulting fusion protein contains the original N-terminal amino acid sequence after its processing by a signal peptidase and its release into the environment.

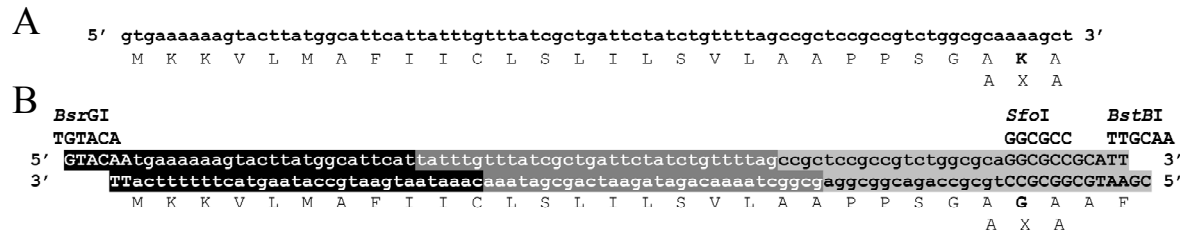


Figure 22: Signal peptide of *B. megaterium* extracellular esterase LipA SP_{lipA}. (A) The DNA sequence and the resulting amino acids of the signal peptide of the extracellular esterase LipA of *B. megaterium* are shown. The cleavage site AXA of the signal peptidase type I is indicated. **(B)** Three pairs of phosphorylated oligonucleotides (indicated by different colours) were hybridised, ligated and cloned into the depicted restriction sites *Bsr*GI and *Bst*BI of pMM1522 placing the coding sequence of the signal peptide under the control of the xylose-inducible promoter P_{xylA}. At the position of the variable amino acid residue X in the indicated signal peptidase cleavage site AXA, SP_{lipA} contained a lysine residue (K). This was changed into a glycine residue (G) creating a new restriction enzyme site for *Sfo*I for direct cloning of target genes into the cleavage site.

For construction of this new protein production and secretion vector, the signal peptide coding sequence consisting of three pairs of annealed synthetic oligonucleotides was cloned into the *Bsr*GI and *Bst*BI restriction sites of pMM1522. The new vector pMM1525 (**Fig. 23**) allows the xylose-inducible production and secretion of proteins of interest in *B. megaterium*. Under control of the promoter P_{xylA}, it encodes the LipA signal peptide followed by a MCS for the in frame insertion of genes of interest. Due to the identity of this MCS between pSTOP1622 and its derivatives (section III.1, **Fig. 14**) and pMM1525 (**Fig. 27**), parallel cloning of genes as translational fusions for intracellular production (series of plasmids named 1622) and for secretion into the growth medium (series of plasmids named 1525) is possible.

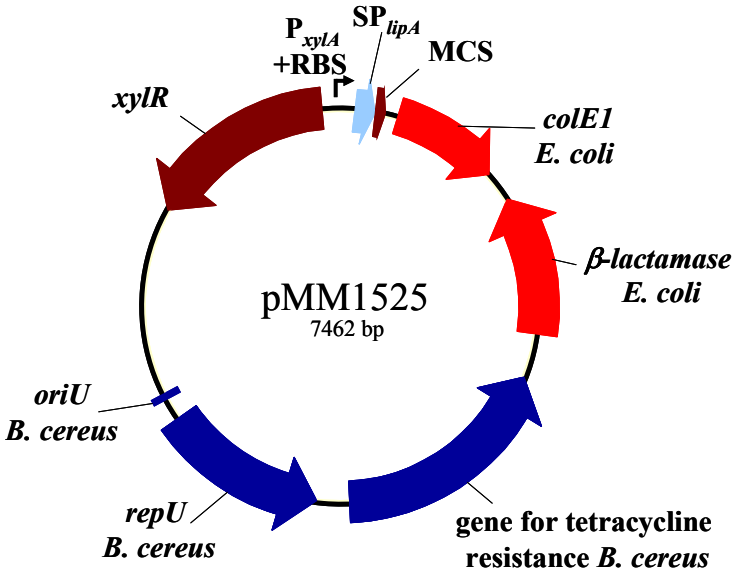


Figure 23: Structure of the *B. megaterium* expression vector pMM1525. Vector pMM1525 is based on the shuttle vector pMM1522. The DNA sequence of the signal peptide of the extracellular *B. megaterium* esterase LipA SP_{lipA} (**Fig. 22**) was inserted into the *Bsr*GI/*Bst*BI digested pMM1522 using the methods of annealed synthetic oligonucleotides. The MCS is located downstream of SP_{lipA} (marked in light blue). Elements for xylose-inducible recombinant gene expression in *B. megaterium* are the inducible promoter (P_{xylA}) and the gene for the xylose repressor (*xylR*, marked in brown). Elements for plasmid replication in *Bacillus sp.* are the *oriU* representing the origin of plasmid replication, *repU*, a gene essential for plasmid replication and the tetracycline resistance gene (all marked in blue). Elements for plasmid replication in *E. coli* are the origin of replication *colE1* and the ampicillin resistance gene *β-lactamase* (both marked in red).

III.2.2 Cloning of *Lactobacillus reuteri* Levansucrase Gene into *Bacillus megaterium* Secretion Vectors

In prior studies, the dextransucrase DsrS from *L. mesenteroides* was used for research on the recombinant production and secretion by *B. megaterium* (Malten *et al.*, 2005a; Malten *et al.*, 2005b). Due to its high relative molecular mass of 188,000 and its tendency to accumulate and form insoluble aggregates it was not a suitable model protein. Hence, the extracellular levansucrase Lev from *L. reuteri* strain 121 was chosen as a new heterologous reporter protein for protein export studies in *B. megaterium*. Full length Lev was found unstable after recombinant production in *E. coli*. A soluble and stable Lev variant called LevΔ773MycHis was previously generated via the deletion of the DNA encoding the N-terminal signal peptide and the C-terminal membrane spanning region and the addition of DNA encoding a C-terminal Myc-epitope and His_6-tag to the *lev* gene (van Hijum *et al.*, 2004). The Myc-epitope and the His_6-tag allow the detection and protein purification of the recombinant protein.

For the secretion of levansucrase LevΔ773MycHis by *B. megaterium*, its complete DNA coding sequence was inserted directly adjacent to the signal peptide coding sequence sp_{lipA} of pMM1525. As described above, the *Sfo*I restriction site allowed the insertion of any desired DNA sequence directly downstream of the signal peptide coding sequence. However, the *Sfo*I restriction endonuclease produces double stranded DNA with blunt ends leading to lower ligation efficiencies. Its isoschizomers *Kas*I and *Nar*I, both creating sticky ends, yielded only partial restriction digests when more than 1 µg of DNA were used. Hence, the *lev∆773*MycHis gene was amplified by PCR and first cloned into the *Sac*I and *Sph*I restriction sites of pMM1525. The *Sac*I restriction site was located downstream of the *Sfo*I site. The employed forward primer carried a *Sac*I site followed downstream by the additional *Sfo*I site at its 5' end, the reverse primer carried a *Sph*I site. The resulting plasmid pMMBm6 contained a linker region between sp_{lipA} and *lev∆773*MycHis flanked by two *Sfo*I sites. This linker was eliminated by *Sfo*I digestion and religation. The resulting pMMBm7 encoded SP_{lipA} directly followed downstream by *lev∆773*MycHis. Positive *E. coli* clones were detected by a translucid colony appearance due to levan production from sucrose after growing them for three days at 30°C on LB medium agar plates containing 5 % (w/v) sucrose and 0.5 % (w/v) xylose for induction of levansucrase gene expression.

III.2.3 Recombinant Protein Production and Secretion by *Bacillus megaterium*

III.2.3.1 Production and Secretion of *Lactobacillus reuteri* Levansucrase by *Bacillus megaterium*

After transformation of pMMBm7 (encoding LevΔ773MycHis) into protoplasted *B. megaterium* MS941 cells, cells were grown aerobically in A5+4 medium. The levansucrase gene expression was induced by the addition of 0.5 % (w/v) xylose at an OD_{578nm} of 0.4. Samples were taken before and 1.5, 3, 4.5, 6 7.5, 9, 10.5, and 12 h after induction of recombinant gene expression and partitioned into fractions of cells and cell-free growth medium by centrifugation. Intracellular proteins were separated into soluble and insoluble fractions while proteins from 1.5 ml growth medium were acetone precipitated and analysed by SDS-PAGE. Significant amounts of an extracellular protein with a M_r of 110,000 were detected (**Fig. 24**, lanes 4-6). As expected, this protein was not present in the growth medium of *B. megaterium* MS941 transformed with the target geneless plasmid pMM1520 (Malten *et al.*, 2005a) (**Fig. 24**, lanes 1-3). As reported previously, the levansucrase LevΔ773MycHis

with a calculated molecular mass of 84,772 Da was observed at a M_r of 110,000 on SDS-PAGE (van Hijum *et al.*, 2001).

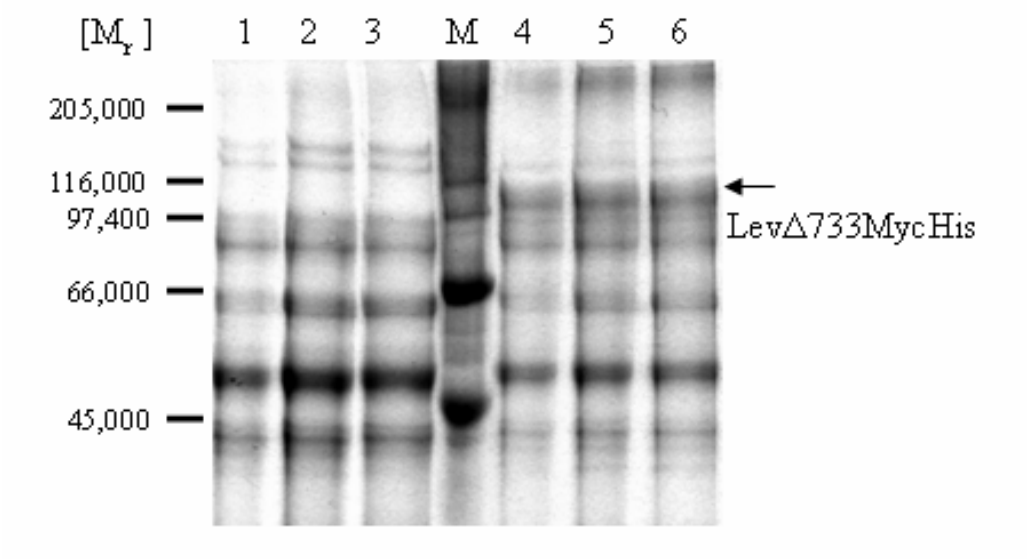


Figure 24: Production and secretion of recombinant *L. reuteri* strain 121 levansucrase LevΔ773MycHis by *B. megaterium* MS941 carrying pMMBm7. *B. megaterium* MS941 transformed with target geneless pMM1520 or pMMBm7 (encoding LevΔ773MycHis) were aerobically cultivated in 100 ml of A5+4 medium at 37°C and 250 rpm. The recombinant gene expression was induced by the addition of 0.5 % (w/v) xylose to the growth medium at an OD_{578nm} of 0.4. Samples were taken at indicated time points and separated into cells and cell-free growth medium by centrifugation. The proteins of 1.5 ml of cell-free growth medium were acetone precipitated, analysed by 12 % SDS-PAGE and stained with Coomassie Brilliant Blue G250. Secreted proteins prepared from MS941 transformed with the target geneless pMM1520 were loaded in lane 1 to 3, while proteins from MS941 transformed with pMMBm7 producing LevΔ773MycHis are shown in lane 4 to 6. Lanes 1/4, 2/5 and 3/6 show proteins prepared 6, 9, and 12 h, respectively, after induction of recombinant gene expression. Lane M: High Molecular Weight Protein Marker (Sigma-Aldrich; St. Louis; USA).

The activity of levansucrase LevΔ773MycHis was initially investigated in an enzyme activity stain after SDS-PAGE analysis of the intra- and extracellular produced proteins (**Fig. 25**). *In situ* levan polymer was formed by active LevΔ773MycHis**.** The *L. reuteri* LevΔ773MycHis produced by *B. megaterium* carrying pMMBm7 (**Fig. 25**, lanes 6-15) and visualised by activity staining had the same relative molecular mass (110,000) as *L. reuteri* LevΔ773MycHis produced by *E. coli* DH10B carrying pBAD-*lev* (**Fig. 25**, lane 2). No such band was visible in the culture medium of *B. megaterium* MS941 transformed with the target geneless pMM1520 (**Fig. 25**, lanes 3-5). The amount of detected polymer formed was proportional to the enzyme concentration (Neubauer *et al.*, 2003).

Levansucrase LevΔ773MycHis was continuously secreted by *B. megaterium* MS941 up to 12 h after induction of the levansucrase gene expression (**Fig. 24**, lanes 3-6). The use of a

protease deficient strain MS941 lacking the major extracellular protease NprM of *B. megaterium* (Wittchen and Meinhardt, 1995) resulted in no levansucrase derived degradation products until 9 h after induction of levansucrase gene expression. Finally, 12 h after induction, a degraded but still active levansucrase form was detectable due to minor extracellular proteases produced under these conditions or due to the start of cell lysis with release of intracellular proteases (**Fig. 25**, lanes 12-15).

1.5 h and 3 h after induction of the levansucrase gene expression the enzyme was barely visible in the cell pellet (**Fig. 25**, lanes 8+10). Interestingly, all cell-associated LevΔ773MycHis was soluble demonstrating that folding problems did not occur during LevΔ773MycHis production and secretion. The cell-associated enzyme had the identical molecular mass as the secreted one. Hence, all cell-associated LevΔ773MycHis was found in the mature form. During SEC-dependent protein secretion, the mature form of secreted proteins is usually exclusively found outside the cytoplasm. Consequently, the residual amount of detected mature cell-associated LevΔ773MycHis protein was located between cytoplasmic membrane and cell-wall. No cytoplasmic levansucrase accumulation was observed which indicated quantitative secretion of the protein into the growth medium.

Additionally to LevΔ733MycHis, a second lower mass protein appeared in the activity stain of A5+4 medium (**Fig. 25**, lanes 5, 12-15). This extracellular enzyme was also present in growth medium after cultivation of the *B. megaterium* MS941 wild type (lane 1) in A5 medium supplemented with 1.0 % (w/v) sucrose. It synthesised a stainable polymer from sucrose classifying it as glycosyltransferase-type enzyme. It will be further considered in section III.3.

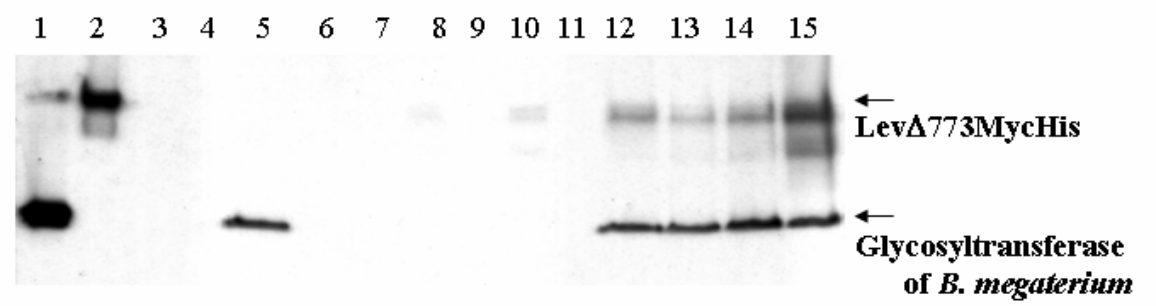


Figure 25: Activity stain for the intra- and extracellular localization of LevΔ733MycHis produced by *B. megaterium* MS941 carrying pMMBm7. Intra- and extracellular proteins prepared from samples taken as described in **Fig. 24** were analysed by 12 % SDS-PAGE. Intracellular proteins were separated into soluble and insoluble fractions and proteins equivalent to 0.5 x 10^9 of cells were analysed. Cell-free growth medium was desalted using PD-10 columns and lyophilised. The dried proteins were solubilised in 8 M of urea and proteins prepared from 750 µl of cell-free growth medium were loaded. After electrophoresis, the gel was washed three times in washing buffer (20 mM sodium acetate pH 5.4, 340 µM $CaCl_2$, 0.1 % (v/v) Triton X-100). Incubation for 36 h at 50°C in washing buffer substituted with 292 mM of sucrose enables the *in situ* formation of polymers by active enzyme. The subsequent oxidation by periodic acid activates the polymer for a stain with Schiff's reagent (Ferretti *et al.*, 1987). Furthermore, *B. megaterium* MS941 was cultivated for 6 h in A5 medium supplemented with 1.0 % (w/v) sucrose. Proteins from 750 µl cell-free growth medium were prepared as mentioned above. As a control, *E. coli* DH10B transformed with pBAD-*lev* encoding levansucrase LevΔ773MycHis were cultivated for 16 h in LB medium supplemented with 100 µg ml^{-1} of carbenicillin and 0.02 % (w/v) arabinose. Cells were opened by lysozyme and benzonase treatment. Proteins equivalent to 0.7 x 10^8 cells were loaded onto the SDS-PAGE gel.

Lane	**Description** (Lane given in brackets)	**Time after induction**
1	Glycosyltransferase-type enzyme secreted by *B. megaterium* MS941 wild type	
2	*L. reuteri* levansucrase intracellularly produced by *E. coli*	
3-5	*B. megaterium* MS941 carrying target geneless pMM1520	
	(3) intracellular soluble; (4) intracellular insoluble; (5) secretome	3 h
6-11	*B. megaterium* MS941 carrying pMMBm7 (LevΔ773MycHis)	
	(6) intracellular soluble; (7) intracellular insoluble;	0 h
	(8) intracellular soluble; (9) intracellular insoluble;	1.5 h
	(10) intracellular soluble; (11) intracellular insoluble	3 h
12-15	Secreted proteins of 1.5 ml of cell-free growth medium from	
	B. megaterium MS941 carrying pMMBm7 (LevΔ773MycHis) (12)	3 h
	(13)	6 h
	(14)	9 h
	(15)	12 h

III.2.3.2 Improved Protein Secretion by *Bacillus megaterium* in Nutrient Rich Medium

B. megaterium MS941 transformed with pMMBm7 (encoding levansucrase LevΔ773MycHis) or target geneless pMM1520 were grown aerobically in A5+4 or LB medium supplemented with 2.5 mM of $CaCl_2$. The levansucrase gene expression was induced by the addition of 0.5 % (w/v) xylose at an OD_{578nm} of 0.4. Samples for measurements of the levansucrase activity in the cell-free growth medium via the D.N.S. (dinitrosalicylic acid) method were taken 6 h after induction of recombinant gene expression. The secretion of LevΔ733MycHis into the growth medium was 4.2 times enhanced by growing *B. megaterium* in LB medium supplemented with $CaCl_2$ compared to the cultivation in A5+4 medium. The nutrient rich LB medium suppressed the export of host exoproteins (**Fig. 24+26**). As a consequence, the recombinant levansucrase was the dominant protein of the secretome. $CaCl_2$ was added to the LB medium because levansucrase was reported to be almost inactive without the addition of calcium cations (Ozimek *et al.*, 2005; van Hijum *et al.*, 2001). The phosphate buffered A5+4 medium already contained 500 μM of $CaCl_2$.

III.2.3.3 Influence of Fusion to Myc-Epitope and His_6-Tag on the Secretion of *Lactobacillus reuteri* Levansucrase by *Bacillus megaterium*

In order to test the influence of the Myc-epitope and the His_6-tag on the secretion of LevΔ773MycHis, both were excluded from the recombinant levansucrase by insertion of a stop codon directly downstream of the levansucrase *levΔ773* coding sequence in pMMBm7 by site-directed mutagenesis. The resulting plasmid pMGBm4 was transformed into protoplasts of *B. megaterium* MS941 cells. *B. megaterium* MS941 carrying pMMBm7 (encoding LevΔ773MycHis) or pMGBm4 (encoding LevΔ773) were cultivated in LB medium supplemented with 2.5 mM of $CaCl_2$. The levansucrase production was induced by the addition of 0.5 % (w/v) xylose to the growth medium at an OD_{578nm} of 0.4. Six h after induction of the levansucrase gene expression, the proteins of 1.5 ml of cell-free growth medium were precipitated by acetone and analysed by SDS-PAGE. Levansucrase LevΔ773 from *B. megaterium* MS941 carrying pMGBm4 possessed a smaller M_r than LevΔ773MycHis (**Fig. 26**). Consequently, the LevΔ773 protein revealed a M_r of 104,000. The elimination of Myc-epitope and His_6-tag increased the amount of secreted LevΔ773 by a factor of 2.8. This was determined by measuring catalytic activities by the D.N.S. method and by quantification of produced levansucrase amounts using Coomassie Brilliant Blue stained SDS-PAGE

(**Fig. 26**). Hence, either the Myc-epitope or the His_6-tag hampered efficient secretion. These observations motivated to further studies on the influence of small epitopes or affinity tags, which are commonly used in protein production and purification, on the secretion efficiency of a fusion protein in *B. megaterium*.

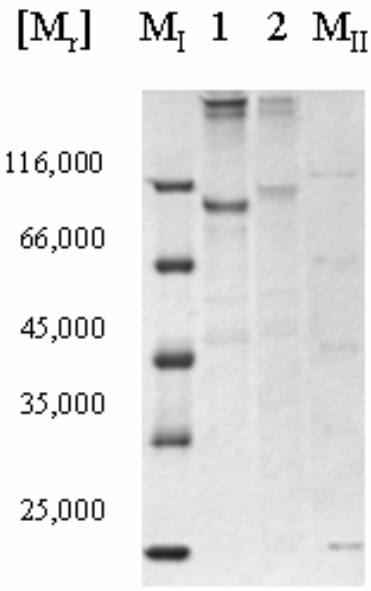


Figure 26: Influence of the Myc-epitope and the His_6-tag on secretion of *L. reuteri* levansucrase LevΔ773 by *B. megaterium*. *B. megaterium* MS941 carrying pMGBm4 (encoding LevΔ773, lane 1) or pMMBm7 (encoding LevΔ773MycHis, lane 2) were aerobically cultivated at 37°C and 250 rpm in 100 ml of LB medium supplemented with 2.5 mM of $CaCl_2$. Six h after induction of the recombinant gene expression at an OD_{578nm} of 0.4 by the addition of 0.5 % (w/v) xylose, the proteins of 1.5 ml of cell-free growth medium were precipitated by acetone, separated by 12 % SDS-PAGE and stained with Coomassie Brilliant Blue G250. Lanes M_I and M_{II} shows Protein Molecular Weight Marker (MBI Fermentas; St. Leon-Rot; Germany) with approximately 750 ng (M_I) or 75 ng (M_{II}) of each protein.

III.2.4 Production and Secretion of Recombinant Affinity Tag Carrying Fusion Proteins

III.2.4.1 *Bacillus megaterium* Expression Vectors for the Extracellular Production of Fusion Proteins with Small Affinity Tags

The intracellular production of the different affinity tagged forms of GFP by *B. megaterium* and their following purification were successful (section III.1). Now, the same two affinity tags, the polyhistidine (His_6-) tag and the StrepII-tag, were chosen to test their influence on the secretion of heterologous levansucrase. For creating a His_6-tag levansucrase fusion, the already introduced pHIS1522 (section III.1) encoding a His_6-tag downstream of the MCS was used. The LipA signal peptide encoding sequence from pMM1525 was inserted upstream of its MCS creating pHIS1525 (**Fig. 27**). This vector allows the insertion of target genes for the production and secretion of C-terminal His_6-tagged fusion proteins. For creating a vector allowing the fusion to a StrepII-tag, the LipA signal peptide coding sequence of pADBm5 was inserted upstream of the MCS of pSTREP1522. This new secretion vector pSTREP1525 (**Fig. 27**) is useful for the production and the secretion of proteins fused to an N-terminal StrepII-tag. This N-terminal StrepII-tag can be removed using the also inserted Factor Xa protease cleavage site.

One affinity tag may be already sufficient for the purification of recombinant proteins. However, systems with two or multiple affinity tags are more frequently found and used. These systems often use one affinity tag at the N-terminus and one at the C-terminus of the target protein. For the purification of some fusion proteins, a single purification step using one type of affinity chromatography does not yield homogenous protein. Here, the use of a second affinity chromatography tag for a second purification step using different column material might help. In order to allow the combination of a target protein with two affinity tags, a *B. megaterium* expression plasmid encoding DNA for an N-terminal StrepII-tag and a C-terminal His_6-tag was constructed. The affinity tag coding sequence of the two secretion vectors pHIS1525 and pSTREP1525 were combined leading to pSTREPHIS1525, an expression plasmid for the extracellular production of StrepII-His_6-tagged proteins (**Fig. 27**). Convenient parallel cloning is possible into all described vectors, when genes of interest are cloned into the identical restriction endonuclease sites of the MCS (*Bgl*II to *Nae*I) (**Fig. 27**).

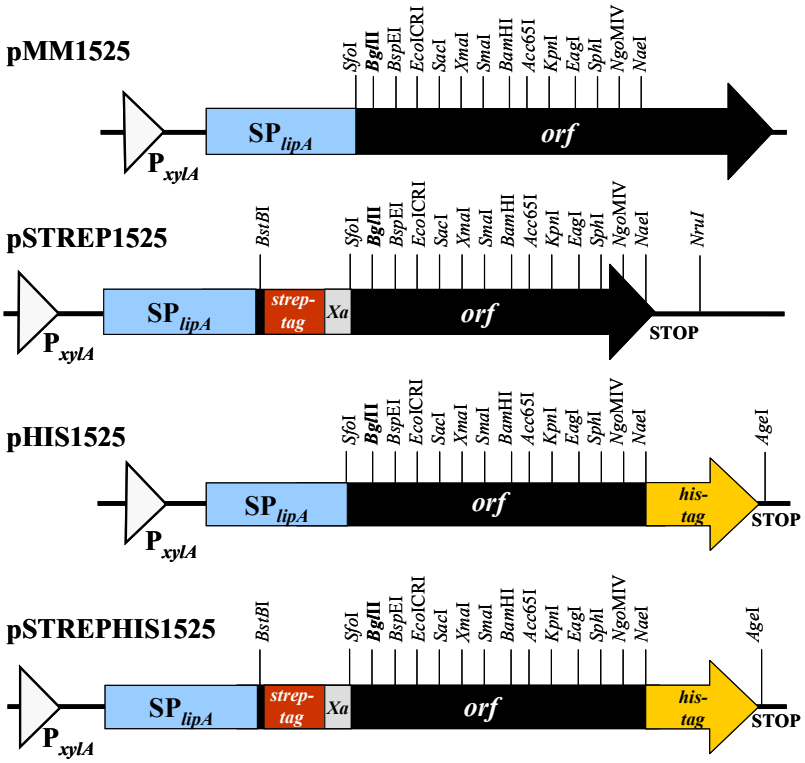


Figure 27: Series of expression plasmids for the secretion of recombinant tagged proteins via SP_{lipA} by *B. megaterium*. All plasmids are based on the shuttle vector pMM1522. The plasmids with the coding sequence for the signal peptide SP_{lipA}, their affinity tag elements and unique restriction enzyme cleavage sites are shown. All shown expression plasmids allow in parallel cloning of genes of interest into the equivalent MCS from *Bgl*II (marked in bold) to *Nae*I.

III.2.4.2 Export of Affinity Tagged Forms of *Lactobacillus reuteri* Levansucrase by *Bacillus megaterium*

The new constructed *B. megaterium* vectors for the secretion of affinity tagged recombinant proteins were now used to further study the effect of a His_6-tag, a StrepII-tag and a Myc-epitope on the secretion of the recombinant levansucrase. Five plasmids encoding different levansucrase fusion proteins were tested. Beside the already introduced pMMBm7 (encoding LevΔ773MycHis – section III.2.2) and pMGBm4 (encoding LevΔ773 – section III.2.3.3), the *levΔ773* gene was cloned into the secretion vectors pSTREP1525, pHIS1525, and pSTREPHIS1525 resulting in pRBBm13 (encoding StrepLevΔ773), pRBBm15 (encoding LevΔ773His), and pRBBm16 (encoding StrepLevΔ773His), respectively. After protoplast transformation, *B. megaterium* MS941 carrying pMMBm7, pMGBm4, pRBBm13, pRBBm15, or pRBBm16, respectively, was cultivated in 100 ml of LB medium supplemented with 2.5 mM of $CaCl_2$. The levansucrase gene expression was induced by the addition of 0.5 % (w/v) xylose to the growth medium at an OD_{578nm} of 0.4. Growth was followed by OD_{578nm} measurements. Samples for protein analysis of cell-free growth medium by Western blot analysis, SDS-PAGE and D.N.S. activity test were taken 6 h after induction of the levansucrase gene expression.

No differences were observed in the growth profiles of all *B. megaterium* plasmid carrying strains. All strains reached the stationary phase 3 h after induction of recombinant gene expression with an OD_{578nm} of around 4.8. In order to investigate the integrity of the exported fusion proteins in the growth medium, the levansucrase fused to the affinity tags was analysed by Western blot experiments using streptavidin conjugate for StrepII-tag detection (IBA GmbH; Göttingen; Germany) and an antibody against the His-tag (GE Healthcare; Uppsala; Sweden) (**Fig. 28A**). Proteins of 1.5 ml of cell-free growth medium were acetone precipitated and analysed after blotting onto PVDF membranes. For StrepLevΔ773, the immunological detection of the StrepII-tag revealed a strong defined protein with an approximate M_r of 108,000 and three minor proteins with an apparent M_r of 97,000, 75,000, and 68,000 (**Fig. 28A**, lane 5). The levansucrase variant carrying two tags resulted in a single protein with an approximate M_r of 110,000 when analysed with the streptavidin conjugate (**Fig. 28A**, lane 4). The anti-His-tag antibody solely detected levansucrase molecules with approximate M_r of 110,000, 106,000, and 110,000 in the growth medium of *B. megaterium* strains carrying pMMBm7 (encoding LevΔ773MycHis, **Fig. 28A**, lane 2), pRBBm15 (encoding LevΔ773His, **Fig. 28A**, lane 3), and pRBBm16 (encoding StrepLevΔ773His, **Fig. 28A**, lane 4), respectively. The degradation products detectable for the N-terminal StrepII-tagged

levansucrase indicated that the protein was partly susceptible to degradation from its C-terminus. Therefore, using an affinity chromatography purification via the C-terminal His_6-tag should lead solely to the intact levansucrase. The Western blot experiments also visualised tagged levansucrase with approximately twice the calculated molecular mass (M_r of 170,000) of the monomer (marked with * in **Fig. 28A**). Increasing the β-mercaptoethanol concentration in the loading buffer from 0.1 to 1 mM abolished this protein dimerisation (data not shown).

After demonstrating the qualitative production and secretion of each fusion protein, the amounts of exported levansucrase by the various tested strains were visualised via SDS-PAGE (**Fig. 28B**). Obviously, the highest amount was found for the strain secreting untagged levansucrase LevΔ773. About half of the amount was observed for strains secreting LevΔ773His, StrepLevΔ773, and LevΔ773MycHis. Even lower amounts were detected for the strain producing StrepLevΔ773His. In order to test the catalytic activities of the different fusion forms of levansucrase LevΔ773, enzyme activity measurements were performed using the D.N.S. method. All produced enzyme variants were found active. Comparison of the protein amounts from the SDS-PAGE analysis with the obtained enzyme activities clearly demonstrated, that the enzyme activities increased in the same magnitude as the detected protein amounts did (**Fig. 28B+C**). In accordance, the specific activity of the affinity chromatographically purified levansucrase variants revealed almost identical values. Hence, enzyme activities measured in triplicates were used to quantitatively compare the amounts of secreted levansucrase LevΔ773 forms. Most levansucrase in the growth medium was detected for the untagged form (57.6 U l^{-1}). Enzyme variants carrying one affinity tag were found exported with less efficiency. LevΔ773His, StrepLevΔ773, and also LevΔ773MycHis revealed about half of the activity of the untagged levansucrase (30.1 U l^{-1}, 25.3 U l^{-1}, and 22.6 U l^{-1}, respectively). In this group of fusion proteins, small but interesting differences were seen in dependence of the size of the fused affinity tag. The His_6-tag consists of 6, the StrepII-tag of 8, and the Myc-epitope plus His_6-tag of 21 amino acid residues. We observed that the longer the amino acid sequence of a tag fused to one end of the protein was, the lower were the detected exported protein amounts. The lowest amount of secreted levansucrase (10.9 U l^{-1}), however, was observed when both StrepII-tag and His_6-tag, with a total of 14 amino acid residues, were added N- and C-terminally.

The influence of small peptide tags on the secretion of heterologous proteins in bacteria has been barely studied. One report compares the secretion of the His_6-tagged with the untagged *B. licheniformis* β-lactamase in *E. coli*. Ledent *et al.* (1997) demonstrated that the C-terminal His_6-tag caused aberrant cleavage of the signal peptide of the investigated β-lactamase.

Hence, this seems to be the first reported study of lower protein secretion efficiency due to a fused small epitope like the Myc-epitope or affinity tag like His_6- and StrepII-tag commonly used in protein production.

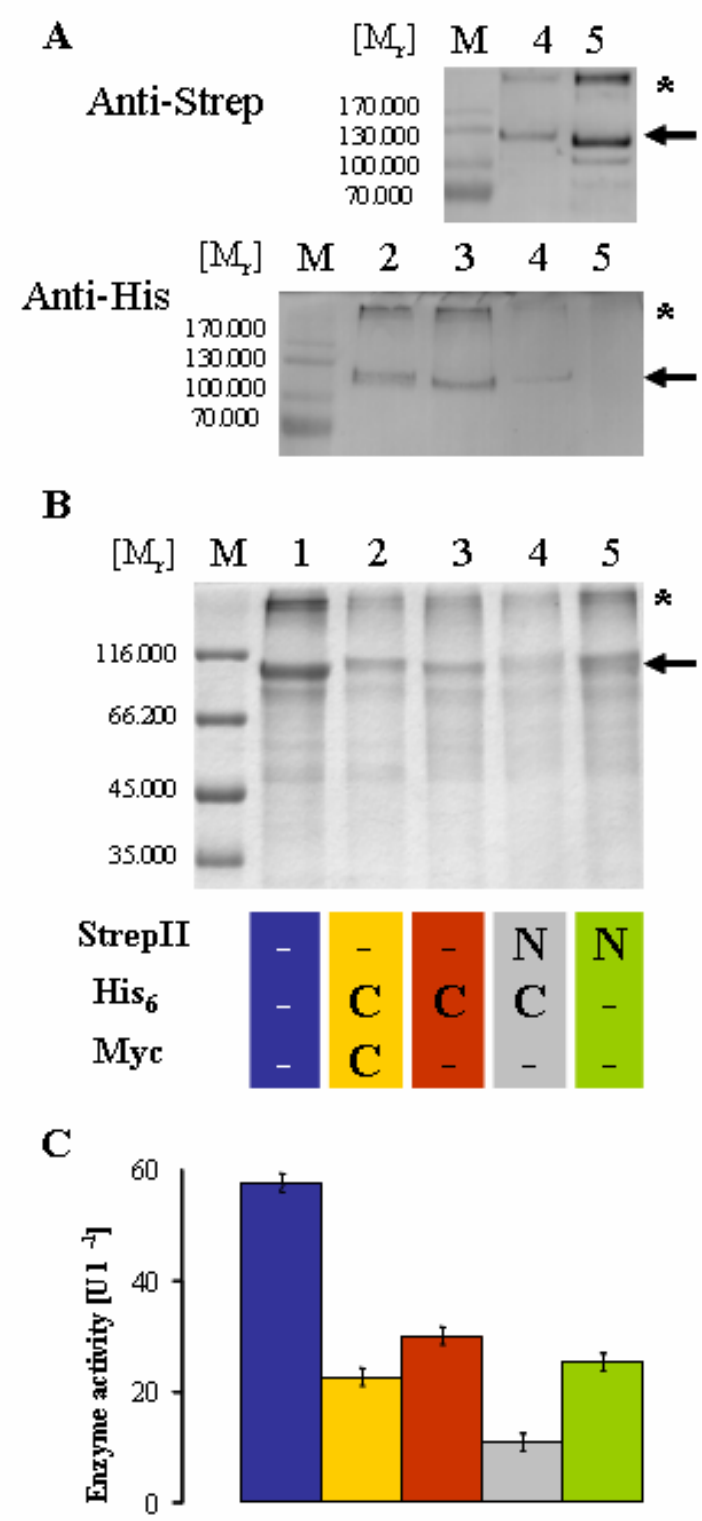


Figure 28: Influence of affinity tags fused to levansucrase LevΔ773 on secretion by *B. megaterium* and enzyme activity. *B. megaterium* MS941 carrying the plasmids encoding LevΔ773 (lane 1), LevΔ773MycHis (lane 2), LevΔ773His (lane 3), StrepLevΔ773His (lane 4) and StrepLevΔ773 (lane 5) were aerobically cultivated in 100 ml of LB medium supplemented with 2.5 mM of $CaCl_2$ at 37°C and 250 rpm. **(A+B)** Six h after induction of recombinant gene expression at an OD_{578nm} of 0.4 by the addition of 0.5 % (w/v) xylose, proteins of 1.5 ml of cell-free growth medium were acetone precipitated and separated by SDS-PAGE. **(A)** The precipitated proteins were blotted onto PVDF membranes and the tags fused to LevΔ773 were detected using streptavidin conjugate or anti-His-tag antibody. Lane M shows PageRuler Prestained Protein Ladder (MBI Fermentas; St. Leon-Rot; Germany). **(B)** The SDS-PAGE gel was stained by Coomassie Brilliant Blue G250. Lane M shows Protein Molecular Weight Marker (MBI Fermentas; St. Leon-Rot; Germany). **(C)** The levansucrase activity in the cell-free growth medium was determined by using the D.N.S. method. One unit of enzyme was defined as the release of 1 µmol glucose per min describing the transferase and hydrolase activity of LevΔ773.

III.2.5 Approaches to Increase the Levansucrase Production and Export by *Bacillus megaterium*

III.2.5.1 Utilisation of a Terminator for Target Gene Transcription

Transcriptional terminators downstream of a gene of interest may sustain its efficient expression. The mRNA stability and thereby protein production can be enhanced by directed transcriptional termination (Makrides, 1996). Plasmid destabilisation due to a transcriptional read through into the replication region is prevented. To investigate the influence of transcriptional termination on protein production in *B. megaterium,* a Rho-independent terminator sequence was introduced into the expression plasmid pRBBm15 downstream of the LevΔ773His coding sequence, resulting in pEJBm7. The employed terminator was previously identified at the 3' end of the *B. megaterium cobI* operon, a 14 gene containing operon important for the synthesis of vitamin B_{12} (Raux *et al.*, 1998). This terminator forms a hairpin loop with an 11 bp stem. It was estimated to have strong termination efficiency. Anyway, no positive effect on the production and secretion of LevΔ773His was observed. To the contrary, the amount of secreted levansucrase 6 h after induction of the levansucrase gene expression measured via the D.N.S. enzyme activity test even slightly decreased to 87 % in the presence of the terminator. Hence, the terminator did not lead to an increase of protein production.

Nevertheless, the existing series of available expression plasmids for the production and secretion of affinity tagged LevΔ773 was supplemented by a plasmid encoding LevΔ773 with a C-terminal StrepII-tag in combination with the terminator. This new plasmid pEJBm2 was constructed cloning synthetic oligonucleotides into pRBBm15 replacing the coding sequence for the His_6-tag. After protoplast transformation of *B. megaterium* MS941 with pEJBm2, cells were grown aerobically in LB medium. It was shown that 4.5 h after induction of recombinant gene expression the maximal amount of secreted LevΔ773Strep was found (41.0 U l^{-1}), which was 1.4-fold less than LevΔ773 (**Fig. 29**), but still 1.4-fold higher than LevΔ773His (**Fig. 28C**).

III.2.5.2 Coexpression of the Extracellular Chaperon Gene *prsA*

In *B. subtilis* the extracellular chaperone PrsA is known to assist protein folding after translocation through cellular membrane via the secretion machinery (Vitikainen *et al.*, 2001). Vitikainen *et al.* showed, that overproduction of PrsA enhanced the secretion of α-amylase of

Bacillus stearothermophilus in *B. subtilis* 4-fold (Vitikainen *et al.*, 2005). Moreover, PrsA overproduction increased the total yield of a secretory single-chain antibody and its secretion by *B. subtilis* 1.6-fold (Wu *et al.*, 1998). In previous studies, a *B. megaterium* expression vector encoding PrsA from *B. subtilis* under control of its own constitutive promoter P_{prsA} was constructed (Malten, 2005). This vector was used for cloning the *levΔ773*MycHis gene under control of the xylose-inducible promoter resulting in pRBBm20. Coexpression of *prsA* with *levΔ773*MycHis showed a negative effect on the secretion of LevΔ773MycHis. No recombinant protein was exported anymore. This phenomenon was also described by Vitikainen and coworkers for the secretion of the endopolygalacturonase PheA of *Erwinia carotovora* by *B. subtilis*. After coexpression of *prsA*, the secretion of this exoprotein into the growth medium decreased (Vitikainen *et al.*, 2005). Secretion of proteins which are folded PrsA-independently in their hosts is not positively or even negatively influenced by the coproduction of PrsA (Vitikainen *et al.*, 2005). So far, no research has been done on the interaction of PrsA and LevΔ773 secretion in *L. reuteri*.

III.2.5.3 Coexpression of Signal Peptidase Gene *sipM*

The *sipM* gene of *B. megaterium* encodes a type I signal peptidase (Nahrstedt *et al.*, 2004). The coexpression of s*ipM* was previously reported to enhance secretion of a recombinant glucanase (Nahrstedt *et al.*, 2004) and a heterologous dextransucrase (Malten *et al.*, 2005b) by *B. megaterium* via a more effective cleavage of the signal peptide. Hence, the signal peptidase gene *sipM* under control of its own constitutive promoter P_{sipM} was cloned into the plasmids encoding LevΔ773MycHis (pMMBm7) and LevΔ773Strep (pEJBm2) resulting in pRBBm19 and pRBBm46, respectively. The levansucrase gene expression was under control of the xylose-inducible promoter. After transformation of these new vectors into protoplasted *B. megaterium* MS941, cells were grown aerobically in LB medium. Three h after induction of the *levΔ773* gene expression the coexpression of *sipM* increased the amount of secreted LevΔ773MycHis and LevΔ773Strep 1.9- and 1.3-fold, respectively (**Fig. 29**).

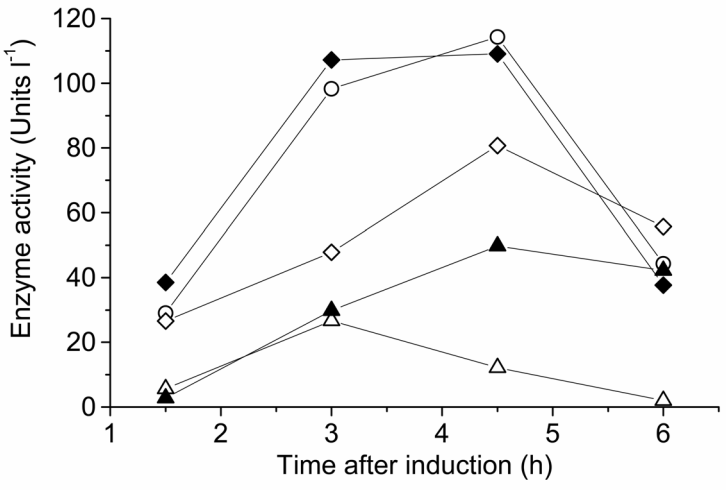


Figure 29: Effect of coexpression of signal peptidase gene *sipM* on secretion of recombinant *L.* levansucrase by *B. megaterium*. Activities measured by the D.N.S. method in the cell-free growth medium of *B. megaterium* MS941 carrying plasmid pMGBm4 (LevΔ773, ○), pMMBm7 (LevΔ773MycHis, Δ), pRBBm19 (LevΔ773MycHis + SipM, ▲), pJEBm2 (LevΔ773 Strep, ◊) and pRBBm46 (LevΔ773Strep + SipM, ♦).

III.2.5.4 Extended Culture Volume and Time

B. megaterium MS941 carrying pRBBm13 (encoding StrepLevΔ773), pRBBm15 (encoding LevΔ773His) or pEJBm2 (encoding LevΔ773Strep), respectively, was cultivated in a 5 times higher volume (500 ml) of LB medium. Furthermore, the cultivation time was extended to 12 h after induction of the recombinant gene expression by the addition of 0.5 % (w/v) xylose at an OD_{578nm} of 0.4. These changes resulted in 21, 14, and 13 times more secreted levansucrase (538 U StrepLevΔ773 per litre, 388 U LevΔ773His per litre, and 523 U LevΔ773Strep per litre, respectively) into the growth medium, respectively. Calculated from the later on determined specific activity (**Fig. 30D**), 2.7 mg of StrepLevΔ773, 2.0 mg of LevΔ773His and 2.7 mg of LevΔ773Strep per litre growth medium were secreted. After growing *B. megaterium* MS941 transformed with the target geneless plasmid pMM1522 under the same conditions, the total protein concentration of the cell-free growth medium after elimination of the small peptides from the complex LB medium by dialysis against sodium phosphate buffer (pH 7.0) was measured as 6.0 mg l^{-1}. Hence, the recombinant tagged LevΔ773 made up to 25 to 30 % of the secreted proteins. Moreover, 4.0 mg l^{-1} of the untagged LevΔ773, equal to 60 % of the total exoproteins, were found secreted into the growth medium.

III.2.6 Affinity Chromatography Purification of Recombinant Secreted Proteins

III.2.6.1 Affinity Chromatographic Purification of StrepII-Tagged LevΔ773 from the Cell-Free Growth Medium

StrepLevΔ773 and LevΔ773Strep were found secreted in high amounts into the growth medium of recombinant *B. megaterium* MS941 strains (**Fig. 29+30A**, lane 1). Hence, the affinity chromatographic purification of StrepLevΔ773 and LevΔ773Strep was tested. Running 50 ml of cell-free growth medium containing StrepLevΔ773 or LevΔ773Strep directly over an 1 ml Strep-Tactin chromatography column led to a rapid light brown colouring of the material. Subsequently, the adsorption of small peptides from the complex growth medium caused a blockage of the column. Therefore, a batch incubation was used. For this purpose, 1 ml of Strep-Tactin sepharose was incubated for 1 h with permanent smooth shaking in 50 ml of cell-free growth medium containing StrepII-tagged LevΔ773. However, only 3 % of StrepLevΔ773 and 0.1 % of LevΔ773Strep protein were found bound to the Strep-Tactin material, respectively. So, only µg amounts of the StrepII-tagged proteins per litre culture broth were purified.

To enhance the binding to the affinity material, StrepLevΔ773 contained in the cell-free growth medium was concentrated by ammonium sulphate precipitation (Englard and Seifter, 1990) prior to purification. This yielded in a 10-fold increase in protein concentration and a recovery of 98 % of native StrepLevΔ773 (**Fig. 30A**, lane 1). In a batch process, the10-fold concentrated solubilised protein precipitate was incubated with 500 µl of Strep-Tactin sepharose. 34 % of the StrepII-tagged levansucrase found in the growth medium were bound to the material. After washing (**Fig. 30A**, lane 2) and elution (**Fig. 30A**, lane 3), 0.7 mg StrepLevΔ773 per litre growth medium were recovered. Beside full length StrepLevΔ773, a few degradation products were also present (**Fig. 30A**). As observed in the Western blot experiments (**Fig. 28A**), a degradation of StrepLevΔ773 occurred from the C-terminus of the protein. Consequently, the presence of the StrepII-tag at the N-terminus also led to binding of shorter degradation forms to the affinity resin. Nevertheless, it was successfully demonstrated that the purification of recombinant StrepII-tagged LevΔ773 directly from the growth medium was possible.

III.2.6.2 Affinity Chromatographic Purification of His_6-Tagged LevΔ773 from Concentrated Cell-Free Growth Medium

The concentration of the cell-free growth medium prior purification of the tagged levansucrase led to a better recovery for StrepLevΔ773. Hence, for purification of LevΔ773His, the cell-free growth medium of 2 litre culture of *B. megaterium* MS941 carrying pRBBm15 (encoding LevΔ773His) was concentrated 25 times via cross-flow filtration using a membrane unit with a cut off value of 50 kDa. SDS-PAGE analysis of the concentrate revealed, that high amounts of LevΔ773His and only small amounts of host cell proteins above a M_r of 45,000 were concentrated (**Fig. 30B**, lane 1). These amounts seemed to correspond to the ratio between heterologous and homologous secreted proteins. Enzyme activities deduced from analysis of appropriately diluted samples of concentrated growth medium (315 U l^{-1}) compared to not concentrated growth medium (388 U l^{-1}) revealed that about 81 % of the 2.0 mg l^{-1} of LevΔ773His were recovered after concentration.

Next, purification of LevΔ773His from concentrated growth medium via column chromatography using Ni-NTA sepharose was tested. 30 ml of 25 times concentrated cell-free growth medium containing LevΔ773His were loaded onto a 200 µl Ni-NTA sepharose column. After washing of the column, the proteins were stepwise eluted. Only 6 % of LevΔ773His were recovered from the concentrated cell-free growth medium. Hence the binding of secreted LevΔ773His to the Ni-NTA affinity material in a buffered solution was tested. Therefore, the concentrated cell-free growth medium was diafiltrated by cross-flow filtration against sodium phosphate buffer (pH 7.0) containing 50 mM, 150 mM, or 300 mM of NaCl. All three buffer conditions yielded high amounts of precipitated proteins. After purification of LevΔ773His from this buffered solution by Ni-NTA sepharose, degradation products of LevΔ773His were observed in the elution fractions as well (data not shown). The degradation might result from the high shear stress in the cross-flow filtration unit. Hence, purification directly from the cell-free growth medium without concentration and diafiltration was pursued.

III.2.6.3 Affinity Chromatographic Purification of His_6-Tagged LevΔ773 from the Cell-Free Growth Medium by Nickel Charged Magnetic Beads

Ji *et al.* (1996) first described the purification of a His-tagged peptide by Ni-NTA super paramagnetic beads. Using this method, the purification of LevΔ773His directly from the cell-free growth medium was tested. From preliminary binding studies using commercially

available paramagnetic pre-charged nickel particles (MagneHis^{TM}; PROMEGA; Madison; USA), a demand of 600 µl of magnetic Ni-particles was calculated for 50 ml of cell-free growth medium containing 2 mg l^{-1} of LevΔ773His. After incubation for 1 h under smooth shaking, the beads were separated from the solution and washed three times with binding buffer. About 1 mg LevΔ773His per litre culture was obtained in the elution steps (**Fig. 30B+D**). Most protein of the expected size was obtained after the first elution step using 100 mM of imidazole (**Fig. 30B**, lane 4). Still considerable amounts of LevΔ773His eluted at higher concentration of imidazole (**Fig. 30B**, lanes 7+8) or remained bound to the magnetic Ni-particles (**Fig. 30B**, lane 9). Hence, a complete dissociation of LevΔ773His from the magnetic Ni-particles was not achieved. The purified LevΔ773His recovered in the first elution step showed a low specific activity of 73.5 U mg^{-1}. Only a 1.1-fold purification with a yield of 16.9 % was calculated from the obtained data (**Fig. 30D**).

III.2.6.4 Purification of His_6-Tagged LevΔ773 from the Cell-Free Growth Medium by Ni-NTA Sepharose in a Batch Process

The observed problem of low recovery was solved using Ni-charged Chelating Sepharose FF (GE Healthcare; Uppsala; Sweden) in a batch process instead of paramagnetic Ni-particles. 700 µl of the chromatographic material were added directly to 50 ml of unconcentrated cell-free growth medium containing LevΔ773His and incubated for 1 h with permanent smooth shaking to increase the contact between the tagged protein and the chromatographic affinity material. After separation of the sepharose from the growth medium, washing (**Fig. 30C**, lane 1) and pre-elution (**Fig. 30C**, lane 2), proteins were eluted using 200 mM of imidazole and 1 mM of β-mercaptoethanol in the binding buffer (**Fig. 30C**, lanes 3-5). This resulted in 0.9 mg of pure LevΔ773His per litre culture medium (**Fig. 30C+D**). The purified protein LevΔ773His of the second main elution step showed a specific activity of 197.4 U mg^{-1}, which is comparable to the 138.0 U mg^{-1} obtained for LevΔ773MycHis produced in *E. coli* (van Hijum *et al.*, 2004). Here, we found a 3.4-fold purification with a yield of 51.5 % (**Fig. 30D**). Hence, for the His_6-tagged levansucrase the batch application of Ni-NTA sepharose in unconcentrated cell-free growth medium was the preferred method for purification. This method was also used later on for the successful purification of a hydrolase from *T. fusca* which was recombinantly produced and secreted by *B. megaterium* (Yang *et al.*, 2006).

III.2.6.5 Comparison of the Purification Procedures for Secreted Fusion Proteins by Ni-NTA or Strep-Tactin Sepharose

For His_6-tagged LevΔ773, no concentration prior to the chromatographic purification was needed which was in contrast to the StrepII-tagged enzyme. One possible explanation might be the significant differences with respect to the affinity parameters of the corresponding tags towards their immobilised binding partners. The dissociation constant (K_D) of the His_6-tag to nickel and of the nickel to NTA are in the range of 10^{-11} and 10^{-9} M, respectively (Dawson *et al.*, 1989). For the StrepII-tag and its binding partner Strep-Tactin a K_D close to 10^{-6} M was measured (Voss and Skerra, 1997) allowing efficient and quantitative binding only in a more concentrated protein solution. Therefore, the complexed nickel containing resin was better suited to capture the His_6-tagged LevΔ773 from a diluted solution like the cell-free growth medium.

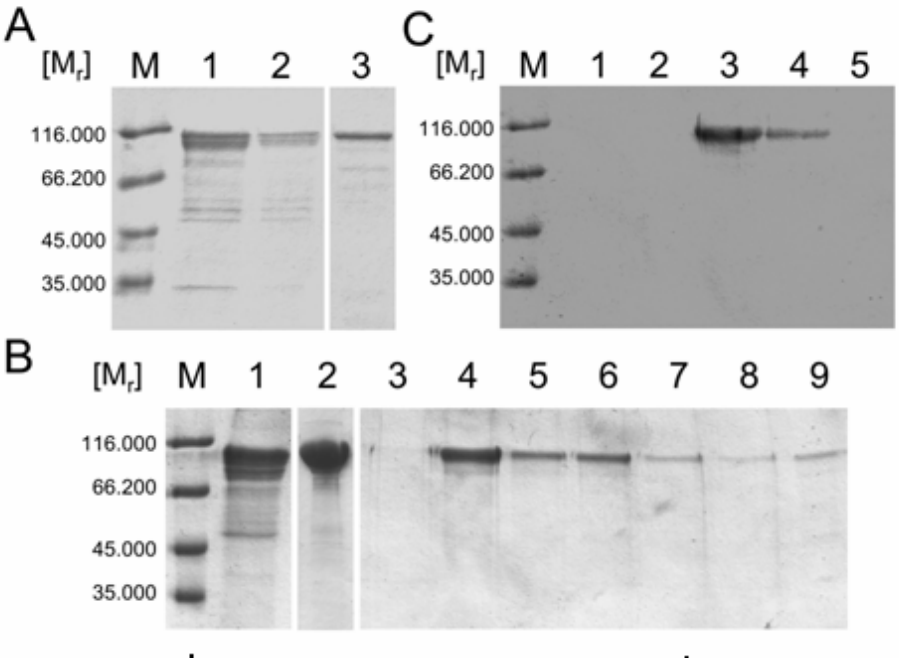
III.2.6.6 Comparison of the Production and Purification of His_6-Tagged LevΔ733 from *Bacillus megaterium* and *Escherichia coli*

Van Hijum *et al.* (2001; 2004) identified, purified and characterised the extracellular levansucrase LevΔ773 from *L. reuteri* after its heterologous production in the commonly used Gram negative bacterium *E. coli*. After this intracellular production of LevΔ773MycHis, the cells had to be disrupted and the cell debris had to be removed from the soluble protein fraction. These steps are time and energy consuming and cost intensive. Production and secretion of His_6-tagged LevΔ773 by *B. megaterium* with the following purification avoided these steps.

The purification from the growth medium was started directly after cell removal. The overall protein concentration in the growth medium was 6.0 mg l^{-1} (**Fig. 30D**). Recombinant LevΔ773His (2.0 mg l^{-1}) was the dominant protein of the *B. megaterium* secretome (**Fig. 30B**, lane 1). For the production of LevΔ773MycHis in *E. coli*, the overall recovered protein amount was 185.6 mg l^{-1} culture medium. In both cases, a Ni-NTA affinity column was used for the protein purification. After affinity purification of His_6-tagged LevΔ773, a specific activity of 197 U mg^{-1} of LevΔ773His produced extracellularly by *B. megaterium* and of 138 U mg^{-1} of LevΔ773MycHis produced intracellularly in *E. coli* were obtained. The employed conditions of the levansucrase assay were comparable in both cases, however, van Hijum *et al.* (2004) directly measured the release of glucose, while here the release of fructose and glucose was measured. Obtained values were converted to the units defined in the

literature (section II.6.11). The more gentle method of the extracellular production and purification of this exoenzyme might be responsible for the higher specific activity observed for the enzyme obtained from *B. megaterium*. In addition, the yield – a measurement for the recovery of total activity after purification compared to total activity prior to purification – was 42.7 % using the *B. megaterium* host compared to only 14.5 % using the *E. coli* system.

Recombinant purified *L. reuteri* levansucrase LevΔ773His produced by *B. megaterium* was further enzymatically characterised. Due to its instability, the purified enzyme could not be stored but had to be directly used for studies on the production of new fructosyloligosaccharides. For this purpose, sucrose analogues such as α-D-galactopyranosyl-1,2-β-D-fructofuranoside (D-Gal-Fru), α-D-xylopyranosyl-1,2-β-D-fructofuranoside (D-Xyl-Fru), α-D-mannopyranosyl-1,2-β-D-fructofuranoside (D-Man-Fru), and α-D-fucopyranosyl-1,2-β-D-fructo-furanoside (D-Fuc-Fru) (Baciu *et al.*, 2005; Seibel *et al.*, 2005; Seibel *et al.*, 2006) were used.



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		B. megaterium: extracellular			*E. coli*: intracellular	
		Unconcentrated growth medium	Ni-NTA magnetic beads (1st Fraction)	Ni-NTA sepharose (2nd Fraction)	Cell extract	Ni-NTA sepharose
Protein	[mg l-1 culture]	6.0	1.0	0.9	185.6	11.8
Specific activity	[U mg-1]	69.5	73.5	197.4	61.0	138.0
Purification	[fold]	1	1.1	2.8	1	2.3
Total activity	[U]	417	70	178	11,322	1,628
Yield	[%]	100	16.9	42.7	100	14.5
Lane		panel B - lane 1 (25x concentrated)	panel B - lane 4	panel C - lane 3		

Figure 30: Affinity chromatography purification of recombinant LevΔ773 from cell-free growth medium of *B. megaterium*. *B. megaterium* MS941 carrying pRBBm13 (encoding StrepLevΔ773) or pRBBm15 (encoding LevΔ773His) were aerobically cultivated in 500 ml of LB medium. 12 h after induction of the *levΔ773* gene expression at an OD_{578nm} of 0.4 by the addition of 0.5 % (w/v) xylose, cells were removed from the growth medium. **(A)** Purification of StrepLevΔ773 using Strep-Tactin sepharose. The proteins of 30 ml of cell-free growth medium were precipitated by ammonium sulphate (lane 1). As a 10-fold concentrate, redissolved proteins were applied onto a 500 µl Strep-Tactin chromatography column. After washing with binding buffer W (lane 2), proteins were eluted with 6 x 250 µl of elution buffer E containing 2.5 mM of desthiobiotin and 1 mM of β-mercaptoethanol (lane 3). **(B)** Purification of LevΔ773His using Ni-charged magnetic beads. Lane 1 shows 20 µl of cell-free growth medium which was 25 times concentrated via cross-flow filtration (GE Healthcare; Uppsala; Sweden). Lane 2 represents proteins bound to 15 µl of Ni-NTA magnetic beads which were added to 10 ml of cell-free growth medium. Lanes 3 to 8 show elution of LevΔ773His bound to 1 ml of Ni-NTA magnetic particles after 1 h of incubation in 50 ml of cell-free growth medium. Proteins from 20 µl of washing fraction (lane 3) and of elution fractions with 1 ml of elution buffer containing 100 mM (lanes 4-6) and 500 mM of imidazole (lanes 7/8) were analysed. Lane 9 shows proteins still attached to 15 µl of Ni charged

magnetic particles after imidazole treatment. **(C)** Purification of LevΔ773His using Ni-NTA sepharose in a batch process. 700 µl of Ni-NTA sepharose were added to 50 ml of cell-free growth medium for 1 h. After separating the affinity material from the growth medium, the material was washed with binding buffer (lane 1). Proteins were eluted in fractions of 300 µl (lane 2), 700 µl (lane 3), 300 µl (lane 4), and three times 650 µl (lane 5) of binding buffer containing 200 mM of imidazole and 1 mM of β-mercaptoethanol. 20 µl of each fraction were analysed via SDS-PAGE. Lane M shows Protein Molecular Weight Marker (MBI Fermentas; St. Leon-Rot; Germany). All SDS-PAGE gels were stained with Coomassie Brilliant Blue G250. **(D)** Comparison of purification of extracellular produced LevΔ773His by *B. megaterium* and intracellular produced LevΔ773MycHis by *E. coli* (van Hijum et al., 2004).

III.2.7 Comparison of Different Signal Peptides for Secretion of *Lactobacillus reuteri* Levansucrase LevΔ773 by *Bacillus megaterium*

In addition to the signal peptide of LipA (SP_{lipA}), the N-terminal fusion of levansucrase LevΔ773 to the signal peptide of the extracellular penicillin G acylase PGA (SP_{pga}) from *B. megaterium* strain ATCC14945 (Panbangred *et al.*, 2000) and to SP_{pga} plus partial as well as the complete α-subunit of PGA was investigated with regard to the influence on secretion of recombinant LevΔ773 by *B. megaterium*. As shown in section III.2.8, recombinantly produced PGA directed by SP_{pga} was secreted in high amounts into the growth medium by *B. megaterium*. The addition of parts or the complete PGA α-subunit was performed in order to evaluate the contribution of the exported protein. Furthermore, a computer aided artificial signal peptide SP_{asp} was tested (**Fig. 31**). This theoretically "optimal" signal peptide was based on the average amino acid sequence of the signal peptides of all Gram positive bacteria. It was constructed by Karsten Hiller in our laboratory using a so called position weight matrix approach implemented in the program PrediSi (Hiller *et al.*, 2004). Based on a systematic database search, the amino acid residue for each position was chosen which occurs most at that position.

SP_{lipA} **MKKVLMAFIICLSLIILSVLAAPPSGAGA AF**
SP_{pac} **MKTKWLISVIILFVFIFPQNLVFA GEDT**
SP_{asp} **MRKRKKALAVALLLAALGALLPTTASA DTFE**

Figure 31: Sequences of the signal peptides SP_{lipA}, SP_{pga} and SP_{asp}. The amino acid sequences of the signal peptides of the *B. megaterium* extracellular lipase (SP_{lipA} – 28 amino acids), the *B. megaterium* extracellular penicillin G acylase (SP_{pga} – 24 amino acids) and of a theoretically designed signal peptide (SP_{asp} – 27 amino acids) are shown with their N-terminal basic, hydrophobic core, and C-terminal domains. The signal peptidase cleavage site is marked as blank, followed by the amino acids aa^{-1} to aa^{-4}.

Secretion vectors pRBBm26 (encoding SP_{pga}), pRBBm27 (encoding SP_{pga} + first 74 aa of the α-subunit of PGA), pRBBm28 (encoding SP_{pga} + first 184 aa of the α-subunit of PGA), pRBBm29 (encoding SP_{pga} + the complete 241 aa of the *α-subunit* of PGA), and pADBm20 (encoding SP_{asp}) (Drews, 2004) were constructed by inserting the coding sequences of the signal peptides and the parts of the α-subunit of PGA into pMM1522. Further, Strep-Xa-l*evΔ773* was cloned into pRBBm26, pRBBm27, pRBBm28, and pRBBm29, resulting in pRBBm30 (encoding SP_{PGA}-Strep-Xa-LevΔ773), pRBBm31 (encoding SP_{PGA}-α-subunit''-Strep-Xa-LevΔ773), pRBBm32 (encoding SP_{PGA}-α-subunit'-Strep-Xa-LevΔ773), and pRBBm33 (encoding SP_{PGA}-α-subunit-Strep-Xa-LevΔ773), respectively. The protein Strep-Xa-LevΔ773 was chosen to allow the removal of parts of the α-subunit from the secreted and purified fusion protein by Factor Xa protease treatment. The *levΔ773*MycHis gene was cloned into pADBm20 resulting in pRBBm24 (encoding SP_{asp}-LevΔ773MycHis).

After protoplast transformation of *B. megaterium* MS941 with pRBBm13 (encoding Strep-Xa-LevΔ773) or with the new plasmids pRBBm30, pRBBm31, pRBBm32, pRBBm33, or pRBBm24, the strains were grown aerobically at 37°C in LB medium supplemented with 2.5 mM $CaCl_2$. Recombinant gene expression was induced at an OD_{578nm} of 0.4 by the addition of 0.5 % (w/v) xylose. Samples for protein analysis via SDS-PAGE, Western blot analysis and D.N.S. activity tests were taken 1.5, 3, 4.5, and 6 h after induction of recombinant gene expression.

For all constructs, maximal levansucrase concentration and activity in the cell-free growth medium was observed 3 h after induction of recombinant gene expression determined by SDS-PAGE, Western blot analysis and activity tests. A densitometric analysis of the SDS-PAGE (data not shown) as well as the D.N.S. activity tests (**Tab. 11**) revealed that the levansucrase fused to SP_{lipA} was secreted best. In contrast, the amount of secreted levansucrase after fusion to SP_{pga} was only 35 %. The fusion to the α-subunit of PGA decreased the amount of secreted levansucrase even further. The longer the α-subunit, the less levansucrase was secreted. This was further proven in Western blot analysis with streptavidin conjugate for the StrepII-tag detection (data not shown).

In *B. subtilis*, many different signal peptides were tested for the secretion of heterologous proteins (Tjalsma *et al.*, 2000). Although clear differences in the efficiency were observed no linkage to the structure of the signal peptide was possible so far. Here, the signal peptide of the *B. megaterium* lipase and the penicillin G acylase differ in their lengths (28 vs. 24 aa) and signal peptidase recognition sites (AGA and VFA, respectively).

Also the secretion of LevΔ773MycHis directed by the artificial signal peptide was successful (62 % compared to LevΔ773MycHis directed by SP_{lipA}). This "optimal" signal peptide (**Fig. 31**) only contains the basic amino acids arginine and lysine in its N-terminal basic domain, whereas the hydrophobic core domain consists of mainly alanine and lysine residues. Moreover, the consensus cleavage site AXA (Tjalsma *et al.*, 1998) is located in the C-terminal domain with an serine residue (S) at the second position. This serine residue is found in most signal peptide type I cleavage sites for *B. subtilis* (Antelmann *et al.*, 2001). The prediction for SP_{asp} was done based on the data of the signal peptides of all Gram positive organisms. The data for the *B. megaterium* leader peptides were not available at that time. Now, based on the genome sequence it might be possible to predict again "the best" signal peptide for the secretion of proteins by *B. megaterium*.

Table 11: Secretion of levansucrase LevΔ773 fused to different signal peptides. The table shows the different fusions of signal peptides to levansucrase LevΔ773. *B. megaterium* MS941 carrying the respective plasmid was grown aerobically in 100 ml of LB medium supplemented with 2.5 mM $CaCl_2$. For all constructs, the highest activity shown as percentage was measured using the D.N.S. activity test 3 h after induction of *levΔ773* gene expression by the addition of 0.5 % (w/v) xylose. All values are normalised to a maximum of 100 % measured for SP_{lipA} dependent secretion of LevΔ773.

Vector	Exported protein	N-terminal fusion	Length of fusion [aa]	Amount of secreted levansucrase [%]
pRBBm13	levansucrase Strep-Xa-LevΔ773	SP_{lipA}	28	100
pRBBm30	levansucrase Strep-Xa-LevΔ773	SP_{pga}	24	35
pRBBm31	levansucrase Strep-Xa-LevΔ773	$SP_{pga}+\alpha''$	102	28
pRBBm32	levansucrase Strep-Xa-LevΔ773	$SP_{pga}+\alpha'$	208	13
pRBBm33	levansucrase Strep-Xa-LevΔ773	$SP_{pga}+\alpha$	265	11
pRBBm24	levansucrase LevΔ773MycHis	SP_{asp}	27	62

III.2.8 Production and Secretion of Additional Recombinant Proteins by *Bacillus megaterium*

III.2.8.1 Production and Secretion of the Penicillin G Acylase PGA by *Bacillus megaterium*

Beside the heterologous levansucrase LevΔ773 from *L. reuteri* 121, the extracellular penicillin G acylase PGA from *B. megaterium* strain ATCC14945 (Panbangred *et al.*, 2000) was chosen as a model protein to investigate the secretion of recombinant proteins. This enzyme is known to catalyse the hydrolysation of penicillin G to phenylacetic acid and β-lactam amino penicillanic acid, a key intermediate for the synthesis of semisynthetic β-lactam antibiotics (Vandamme and Voets, 1974). Furthermore, it forms novel semisynthetic β-lactam antibiotics in the reverse reaction (Yang *et al.*, 2001). Here, PGA was recombinantly produced and secreted by *B. megaterium* strain MS941. Therefore, *pga* including the coding sequence of its natural signal peptide SP_{pga} (section III.2.7) was cloned into the vector pMM1522. The resulting vector pRBBm23 was transformed into protoplasted *B. megaterium* MS941 cells.

B. megaterium MS941 carrying pRBBm23 (encoding SP_{pga}-PGA) and for comparison MS941 carrying pMMBm7 (SP_{lipA}-LevΔ773MycHis) were grown aerobically in 100 ml of LB medium. Recombinant gene expression was induced at an OD_{578nm} of 0.4 by the addition of 0.5 % (w/v) xylose. Growth was followed by OD_{578nm} measurements showing comparable growth profiles for both strains. Six h after induction of recombinant gene expression, samples for the analysis of the extracellular proteins were taken. After cell removal, acetone precipitated proteins of 1.5 ml of cell-free growth medium were analysed by SDS-PAGE (**Fig. 32)**. After secretion, PGA is autocatalytically cleaved into an α- and a β-subunit with a M_r of 27,000 and 57,000, respectively (Panbangred *et al.*, 2000). Comparing the amounts of penicillin G acylase PGA and levansucrase LevΔ773MycHis detected on an SDS-PAGE gel, clearly demonstrated that PGA directed by its natural signal peptide SP_{pga} was secreted in higher amounts.

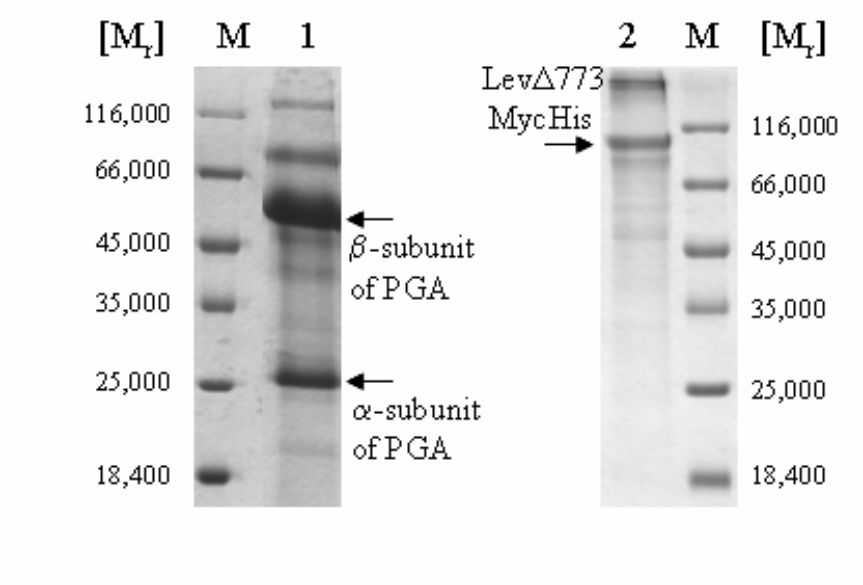

Figure 32: Comparison of PGA and LevΔ773MycHis production by *B. megaterium*. *B. megaterium* MS941 transformed with pRBBm23 (encoding SP_{pga}-PGA, lane 1) or pMMBm7 (encoding SP_{lipA}-LevΔ773MycHis, lane 2) were cultivated aerobically in 100 ml of LB medium at 37°C and 250 rpm. The recombinant gene expression was induced by the addition of 0.5 % (w/v) xylose to the growth medium at an OD_{578nm} of 0.4. Six h after induction of recombinant gene expression, the proteins of 1.5 ml of cell-free growth medium were precipitated by acetone, analysed by 12 % SDS-PAGE and stained with Coomassie Brilliant Blue G250. Lanes M show Protein Molecular Weight Marker (MBI Fermentas; St. Leon-Rot; Germany).

III.2.8.2 Coexpression of the Extracellular Chaperon *prsA* or Signal Peptidase *sipM*

After testing the contribution of a coexpression with the gene for the extracellular chaperon *prsA* and the gene for the signal peptidase *sipM* (sections III.2.5.2 and III.2.5.3), respectively, with the target gene *levΔ773*, the same approach was followed for the *pga* gene. The combination of the *prsA* gene under control of its own constitutive promoter P_{prsA} and the *pga* gene under control of the xylose-inducible promoter P_{xylA} was realised using vector pRBBm18. After transformation of pRBBm18 into protoplasted *B. megaterium*, cells were grown aerobically in LB medium. Analysis of the secreted proteins after induction of recombinant gene expression showed a negative effect of the PrsA production on the secretion of PGA. Almost no recombinant PGA was secreted anymore as demonstrated by SDS-PAGE analysis and by the NIPAB (6-Nitro-3-phenylacetamido-benzoic acid) enzyme assay which was used to detect PGA activity in the cell-free growth medium (data not shown). Like the secretion of recombinant LevΔ773, the secretion of PGA seemed to be PrsA-independent while the decrease of the secretion remains unclear (Vitikainen *et al.*, 2005).

Coexpression of levansucrase gene *levΔ773*MycHis with the *sipM* gene resulted in an increase of levansucrase export (section III.2.5.3). Hence, P_{sipM}-*sipM* was cloned into pRBBm23 (P_{xylA}-*pga*) resulting in pRBBm17. After transformation of pRBBm17 into protoplasted *B. megaterium* MS941, the plasmid carrying strains were grown aerobically in LB medium. Recombinant gene expression was induced at an OD_{578nm} of 0.4 by the addition of 0.5 % (w/v) xylose. 3, 6, and 9 h after induction of recombinant gene expression, samples for the analysis of the extracellular proteins by SDS-PAGE and for NIPAB activity assays were taken. After cell removal, acetone precipitated proteins of 1.5 ml of cell-free growth medium were analysed via SDS-PAGE (data not shown). No differences were detected in the amount of PGA with or without coproduction of SipM on the SDS-PAGE gel. This was confirmed by the NIPAB activity test (data not shown). Hence, co-production of SipM had no influence on the *per se* strong secretion of PGA. This fact indicates, that signal peptide cleavage does not presents a bottle neck in the secretion of PGA.

III.3 Alternative Promoter Systems in *Bacillus megaterium*

III.3.1 Use of the IPTG-Inducible Promoter P_{spac} for Recombinant Gene Expression in *Bacillus megaterium*

As already demonstrated in shaking flask and in high cell density cultivations, the xylose-inducible promoter P_{xylA} used for recombinant protein production in *B. megaterium*, was tightly controlled and led up to 274 mg of recombinantly produced GFP per litre culture. Beside this xylose-inducible promoter, alternative promoter systems were to be tested. Other inducible promoters are of great interest for the two vector system introduced in III.1.6. Hence, the IPTG-inducible promoter P_{spac} was investigated. The P_{spac} promoter is a widely used promoter for the expression of recombinant genes in *E. coli* and *B. subtilis*. Unfortunately, this promoter is not tightly controlled which makes it difficult to use for the production of toxic proteins.

Anyway, for testing this promoter in *B. megaterium*, the origin of replication *oriU* and the *repU* gene of pMM1522 were cloned into pMUTIN-GFP+ (BSGC; USA), a shuttle vector for cloning in *E. coli* and for genomic integration as well as P_{spac} controlled the *gfp* gene expression in *B. subtilis*. The new *B. megaterium* vector named pRBBm40 encoded the sequence of P_{spac}, the *trpA*-terminator downstream of the *gfp* gene and the repressor LacI. After protoplast transformation of *B. megaterium* MS941 with pRBBm34 (encoding P_{xylA}-GFP – section III.1.6.1) or pRBBm40 (encoding P_{spac}-GFP), cells were grown aerobically in LB medium at 37°C. At an OD_{578nm} of 0.4, the *gfp* gene expression was induced by the addition of 0.5 % (w/v) xylose in MS941 carrying pRBBm34 or with 500 µM of IPTG in MS941 carrying pRBBm40. 3, 6, 8, and 22 h after induction of the *gfp* gene expression samples for spectroscopic GFP measurements were taken. Negative controls were *B. megaterium* MS941 carrying pSTOP1622 as well as MS941 carrying pRBBm34 (encoding P_{xylA}-GFP) or pRBBm40 (encoding P_{spac}-GFP) without induction of the *gfp* gene expression.

Only very weak fluorescence signals were detected of *B. megaterium* MS941 carrying pRBBm40 (encoding P_{spac}-GFP) with and also without induction of the *gfp* gene expression via IPTG addition. Hence, the P_{spac} promoter did not seem to be controlled by the addition of IPTG in *B. megaterium*. No fluorescence of *B. megaterium* MS941 carrying target geneless pSTOP1622 or pRBBm34 (encoding P_{xylA}-GFP) without xylose addition were measured while the typical GFP derived fluorescence spectra were detected testing MS941 carrying pRBBm34 (encoding P_{xylA}-GFP) after induction of the *gfp* gene expression with xylose.

III.3.2 A Sucrose Inducible Promoter of *Bacillus megaterium*

The *B. megaterium* genome itself was investigated for alternative strong and inducible promoters. For this purpose, *B. megaterium* wild type strain DSM319 was subjected to various environmental stimuli (oxygen supply, osmotic pressure) and different nutrient sources (C-, N-, P-source, $CaCl_2$, oil) in LB or minimal medium. At different time points after the cultivation start, the medium was separated from the cells, secreted proteins were precipitated and analysed by one and two dimensional PAGE. The gels were screened for dominant protein bands and spots which represented strongly secreted proteins under the analysed growth conditions.

Growing *B. megaterium* DSM319 in the presence of sucrose, a dominant band representing a protein with a M_r of 52,000 was detected via SDS-PAGE (**Fig. 33**). It was not present in the control experiment without sucrose addition. Based on the 2D PAGE, an isoelectric point between 5.0 and 5.5 was determined. The corresponding protein band was identified as a levansucrase (MALDI-TOF/MS analysis, L. Jänsch; Helmholtz Centre for Infection Research; Braunschweig; Germany) with 73 % identity of the amino acid sequence to the levansucrase SacB of *B. subtilis* and 71 % identity to the levansucrase SacB of *B. amyloliquefaciens*. Therefore, it was also named levansucrase $SacB_{B.meg}$. Due to the strong secretion of $SacB_{B.meg}$, *B. megaterium* showed reduced growth. Only half of the cell densities were achieved compared to the strain without addition of sucrose. This behaviour is common and often described for strains producing high amounts of proteins (Glick, 1995).

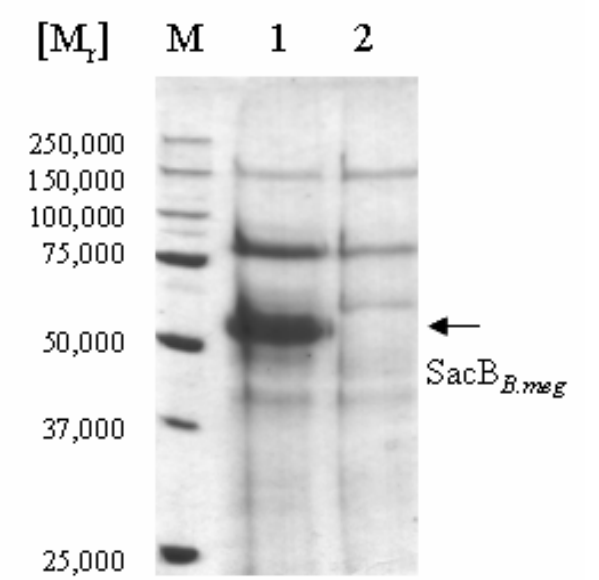

Figure 33: Effect of sucrose on the secretome of *B. megaterium* DSM319. *B. megaterium* DSM319 was cultivated aerobically in 100 ml of LB medium with (lane 1) or without (lane 2) the addition of 0.5 % (w/v) sucrose at 37°C and 250 rpm. Six h after cultivation start, 1.5 ml of cell-free growth medium were desalted using a PD-10 column (GE Healthcare; Uppsala; Sweden) and subsequently lyophilised. The precipitated proteins were analysed via 10 % SDS-PAGE and stained with Coomassie Brilliant Blue G250. Lane M shows Precision Plus Protein Standard (Bio-Rad; Munich; Germany).

III.3.2.1 Regulation of the Sucrose Dependent Secretion of the Levansucrase SacB in *Bacillus subtilis*

Global regulatory responses allow *B. subtilis* to adapt efficiently to changes in the environmental conditions. These responses often include changes in the transcription rates of large sets of target genes and thus, control a wide variety of cellular processes. One of this processes is the synthesis of degradative enzymes like the levansucrase SacB (Steinmetz, 1993). The enzyme is well characterised and already crystallised (Chambert and Petit-Glatron, 1991; Meng and Futterer, 2003). The secretion of the *B. subtilis* SacB is controlled by the two component systems DegS/DegU and as well as SacX/SacY. The gene s*acB* presents the first open reading frame in an operon followed by a gene encoding a levanase. The intergenic noncoding region upstream of the start codon of *sacB* consists of 540 bp. The promoter of this operon P_{sacB}, also called *sacR*, is located 230 bp upstream of the start codon of *sacB* (Aymerich *et al.*, 1986). Between promoter and ribosome binding site, a 66 bp region forms a hairpin structure at the RNA level (ribonucloetide antiterminator sequence RAT) to which the transcriptional antiterminator SacY binds. SacY is negatively regulated by the regulatory protein SacX (Crutz and Steinmetz, 1992; Idelson and Amster-Choder, 1998). Both genes, *sacX* and *sacY*, are located in an operon controlled by the two component system DegS/U and SacY. SacY shows a RNA-binding as well as a sucrose-binding site (Manival *et al.*, 1997). Hence, it controls its own and *sacB* transcription (Tortosa and Le Coq, 1995). The sensor histidine kinase DegS phosphorylates the response regulator DegU which positively regulates the *sacB* gene expression by binding to its promoter as well as to the promoter of the *sacX/Y* operon. DegQ, a 46 amino acid residue transcription factor which is involved in the production of degradative proteins in *B. subtilis* (Amory *et al.*, 1987), further stimulates *sacB* transcription.

The potential genes encoding proteins homologous to DegS/DegU as well as for SacX/SacY were found in the genome of *B. megaterium*, but no DegQ homolog was found so far. In *B. megaterium*, $sacB_{B.meg}$ was found as the first gene of a potential operon with the second gene encoding a levanase like in *B. subtilis*. The intergenic region upstream of *sacB* in *B. megaterium* consists of nearly 600 bp. The comparison of the promoter regions of *B. subtilis* and *B. megaterium sacB* failed to detect obvious homologies.

III.3.3 Comparison of the Sucrose ($P_{sacBB.meg}$) and the Xylose (P_{xylA}) Inducible Promoters for Recombinant Gene Expression in *Bacillus megaterium*

For initial promoter studies, the whole 600 bp intergenic region upstream of $sacB_{B.meg}$ including the ribosome binding side and the coding sequence of the 10 first amino acid of SacB *sacB'* of *B. megaterium* strain DSM319 were amplified. This DNA-fragment was cloned into pRBBm34 (encoding P_{xylA}-GFP; **Fig. 34B**), replacing *xylR* and P_{xylA}. The new plasmid was named pRBBm59 (encoding $P_{sacBB.meg}$-GFP; **Fig. 34C**). Plasmid pRBBm59 was transformed into protoplasted *B. megaterium* WH323. Plasmid strains WH323 carrying pRBBm34 (encoding P_{xylA}-GFP) and WH323 carrying pRBBm59 (encoding $P_{sacBB.meg}$-GFP) were grown aerobically in 50 ml of LB medium at 30°C or 37°C. The recombinant *gfp* gene expression was induced at an OD_{578nm} of 0.4 by the addition of 0.5 % (w/v) xylose and of 0.5 % (w/v) sucrose, respectively. Cultivations of both plasmid carrying strains without inducer addition served as control. Samples for OD_{578nm} measurements and GFP analysis by fluorescence spectroscopy were taken 0.5, 1.5, 3, 4.5, 6, and 7.5 h after induction of the *gfp* gene expression. The plasmid carrying strains showed differences in their growth behaviour. WH323 carrying pRBBm59 (encoding $P_{sacBB.meg}$-GFP) reached the stationary phase 1.5 h after induction of the *gfp* gene expression with sucrose with an OD_{578nm} of only 2.4, while WH323 carrying pRBBm34 (encoding P_{xylA}-GFP) reached the stationary phase with a final OD_{578nm} of 4.2 between 4.5 h and 6 h after induction of the *gfp* gene expression with xylose. Cultivations of both strains without addition of inducer were equal in their growth profiles, reaching the stationary phase also after 4.5 h and 6 h with a final OD_{578nm} of 4.2.

For quantification of GFP production, cells were separated from the growth medium and diluted in sodium phosphate buffer to a final concentration of 10^9 of cells per ml. At a growth temperature of 30°C, no differences were observed in the maximal amounts of formed GFP between plasmid strain WH323 carrying pRBBm34 (encoding P_{xylA}-GFP) and WH323 carrying pRBBm59 (encoding $P_{sacBB.meg}$-GFP). Six h after induction of the *gfp* gene expression around 2.2 µg GFP per OD were detected. At a growth temperature of 37°C maximal GFP production was measured 4.5 h after induction of the *gfp* gene expression. Here, higher GFP production was detected if the *gfp* gene expression was under control of the promoter $P_{sacBB.meg}$ (2.73 µg GFP per OD) compared to P_{xylA} (2.26 µg GFP per OD). No GFP production was measured for the strain WH323 carrying pRBBm34 (encoding P_{xylA}-GFP) without xylose addition. However, 0.62 µg GFP per OD where present during the whole cultivation time in strain WH323 carrying pRBBm59 (encoding $P_{sacBB.meg}$-GFP). Similarly, a basal promoter activity of vector encoded *B. subtilis* promoter P_{sacB} in *B. subtilis* was

described (Kunst and Rapoport, 1995). Here, coexpression of *degQ* encoding a transcription factor that stimulates transcription of the *sacB* promoter in *B. subtilis* led to a stringent control of the promoter in the absence of sucrose. In the presence of sucrose, the coexpression of *degQ* led to a strong increase of recombinantly produced staphylokinase and TEM β-lactamase (Wong, 1989; Ye *et al.*, 1999).

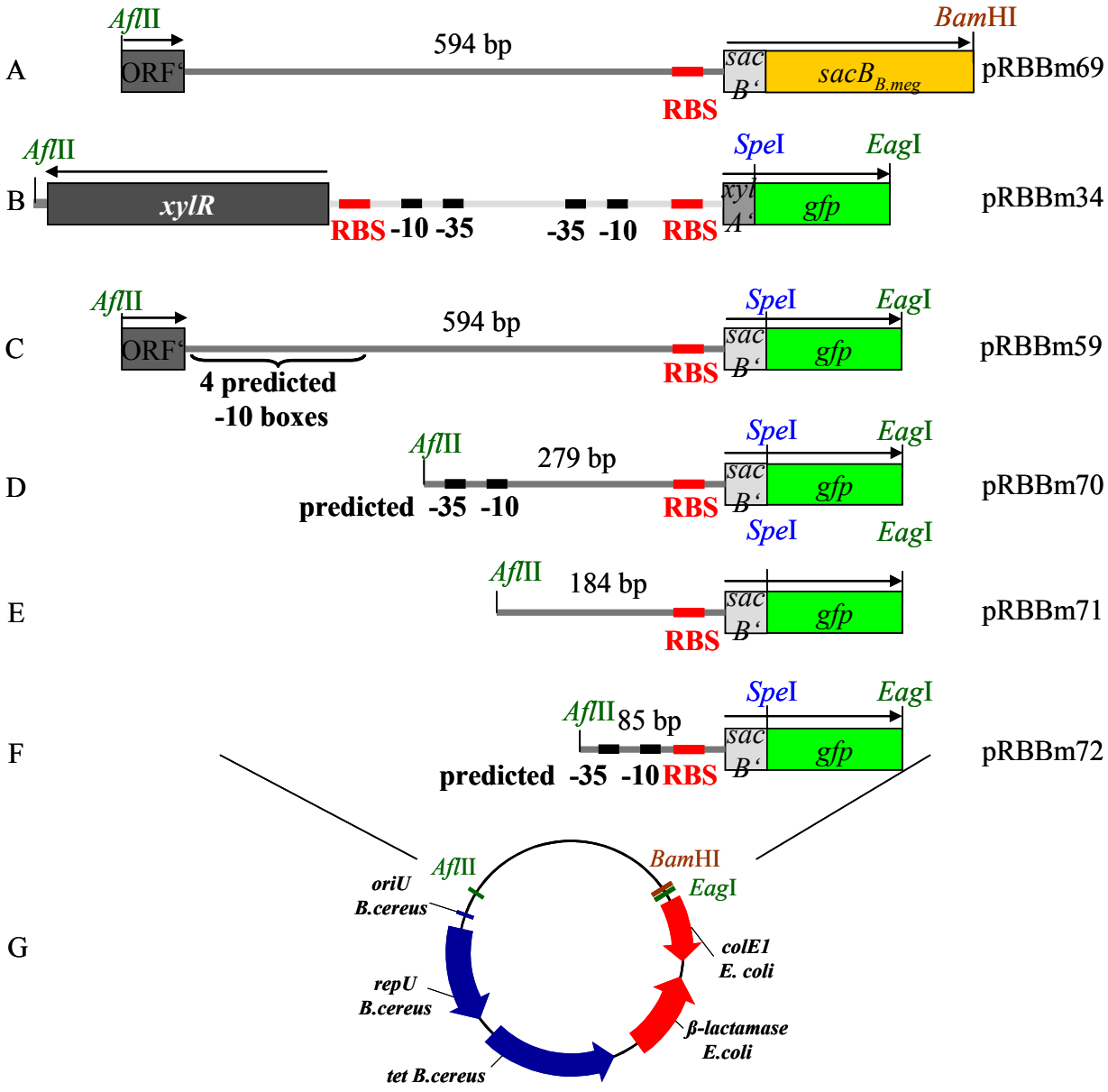

Figure 34: Mutational analysis of the *B. megaterium* sucrose inducible promoter P_{sacB}. Genetic organisation of the elements responsible for recombinant *gfp* gene expression using pRBBm69 **(A)**, pRBBm34 **(B)**, pRBBm59 **(C)**, pRBBm70 **(D)**, pRBBm71 **(E)**, and pRBBm72 **(F)** is shown. For pRBBm59 **(C)**, pRBBm70 **(D)**, pRBBm71 **(E)**, and pRBBm72 **(F)**, the potential promoter P_{sacB} was cloned into pRBBm34 **(B)** via *Afl*II and *Spe*I replacing *xylR* and P_{xylA}. Fragments **A – F** are located in the basis shuttle vector of the 1622 series (section III.1.1) between the indicted restriction sites *Afl*II and *Bam*HI or *Eag*I. **(G)** Basis shuttle vector of the 1622 series. Elements for plasmid replication in *Bacillus sp.* are the *oriU* representing the origin of plasmid replication, *repU*, a gene essential for plasmid replication

and the tetracycline resistance gene (all marked in blue). Elements for plasmid replication in *E. coli* are the origin of replication *colE1* and the ampicillin resistance gene *β-lactamase* (both marked in red). **(A-G)** The *Afl*II and *Eag*I restriction sites are marked in green, *Spe*I restriction site is marked in blue while *Bam*HI restriction site is marked in brown. RBS – potential ribosome binding site; -10 and -35 – potential -10 and -35 elements of the promoter $P_{sacBB.meg}$; *gfp* – coding sequence of GFP; *xylA'* – coding sequence of the first 4 amino acids of XylA; *sacB'* – coding sequence of the first 10 amino acids of $SacB_{B.meg}$. Open reading frames are indicated by arrows. **(A, C-F)** Distances between *Afl*II restriction site and *sacB'* start codon are given in base pairs (bp).

III.3.4 Mutational Analysis of the *Bacillus megaterium* Promoter $P_{SacBB.meg}$

The functionality of the sucrose inducible promoter P_{sacB} using GFP as model protein was successfully demonstrated. Now, a more detailed promoter analysis was demanded. Bioinformatic tools were used to identify the typical -10 (TATA) box of this promoter. "Virtual Footprint" (http://www.prodoric.de/vfp/vfp_promoter.php) is suited for the recognition of corresponding DNA patterns. It was designed for the analysis of transcription factor binding sites and their underlying regulatory networks in whole bacterial genomes. The pasted DNA-sequences are scanned for binding sites of regulators from many different organisms. With the exception of the catabolite controlled protein (CcpA) (Deutscher *et al.*, 1995), no experimental information for the binding site of *B. megaterium* regulators are currently available. Hence, using the binding site for the sigma 70 factor of *E. coli* for the theoretical detection of potential -10 boxes in the promoter region upstream of $sacB_{B.meg}$ revealed six potential -10 boxes. Based on this results, three vectors with continuously shortened promoter regions where constructed (**Fig. 34D-F**). Four of the potential -10 boxes were solely included in the largest 600 bp fragment encoded on pRBBm59 (**Fig. 34C**). Promoter regions including the ribosome binding side and the coding sequence of the first 10 amino acid of SacB *sacB'* of *B. megaterium* DSM319 with lengths of 361 bp, 266 bp, and 167 bp were amplified and cloned into pRBBm34 (encoding P_{xylA}-GFP; **Fig. 34B**) replacing *xylR* and P_{xylA}. The new vectors were named pRBBm70, pRBBm71, and pRBBm72, respectively. Vector pRBBm70 encoded 279 bp, pRBBm71 184 bp and pRBBm72 85 bp upstream of the start codon of $sacB_{B.meg}$ (**Fig. 34D-F**). Beside the sigma 70 binding sites, "Virtual Footprint" calculated the stacking energy of the DNA region. This is reciprocally proportional to the GC-content. The promoter of the *sacB* gene of *B. subtilis* is situated 230 bp upstream of the ATG of *sacB* (Aymerich *et al.*, 1986). For *B. megaterium* the highest peak of the stacking energy was found around 30 bp upstream of the start codon of $sacB_{B.meg}$. A corresponding short promoter construct is encoded on pRBBm72.

After protoplast transformation of *B. megaterium* strain WH323 with these new plasmids, all new plasmid carrying strains were grown aerobically in 50 ml of LB medium at 37°C. Recombinant *gfp* gene expression was induced by the addition of 0.5 % (w/v) sucrose at an OD_{578nm} of 0.4. Samples for OD_{578nm} measurements and fluorescence spectroscopy were taken 0.5, 1.5, 3, 4.5, 6, and 7.5 h after induction of the *gfp* gene expression. All plasmid carrying strains showed indentical growth profiles reaching the stationary phase 1.5 h after induction of the *gfp* gene expression with an final OD_{578nm} of 2.4. For quantification of GFP, cells were separated from the growth medium by centrifugation and diluted in sodium phosphate buffer to a final concentration of 10^9 of cells per ml. No GFP production was detectable for strain WH323 carrying pRBBm72 representing the 85 bp long promoter construct. Moreover, only 12.5 % GFP were produced by strain WH323 carrying pRBBm70 (184 bp promoter construct) and WH323 carrying pRBBm71 (279 bp promoter construct), respectively, compared to the production of strain WH323 carrying pRBBm59 (594 bp promoter fragment). Consequently, even the 279 bp of the intergenic region upstream of the *sacB* start codon did not include all positive regulator elements of the complex $P_{sacBB.meg}$ promoter.

III.3.5 Enhanced Levansucrase $SacB_{B.meg}$ Production by Increasing the $P_{sacBB.meg}$-$sacB_{B.meg}$ Copy Number in *Bacillus megaterium* Using a Multi Copy Plasmid

The copy number of $P_{sacBB.meg}$-$sacB_{B.meg}$ was increased by cloning the 594 bp intergenic region upstream of $sacB_{B.meg}$ followed by the levansucrase $sacB_{B.meg}$ gene itself into pMM1522 replacing the elements for the xylose-inducible gene expression. The novel vector was named pRBBm69 (encoding $P_{sacBB.meg}$-$sacB_{B.meg}$, **Fig. 34A**). Now, one can quantify the effect of multi copy expression of levansucrase *sacB* gene over the background of a chromosomal located copy of *sacB* gene. After protoplast transformation of *B. megaterium* MS941, the plasmid strains MS941 carrying pRBBm69 and MS941 carrying the target geneless pMM1520 were grown aerobically in 100 ml of LB medium at 37°C. The $sacB_{B.meg}$ expression was induced by the addition of 0.5 % (w/v) sucrose at an OD_{578nm} of 0.4. Samples for OD_{578nm} measurements and D.N.S. activity tests for quantifying the amount of secreted levansucrase $SacB_{B.meg}$ were taken 0.5, 2, 3, 4.5, and 6 h after induction of $sacB_{B.meg}$ expression. No differences were observed in the growth profiles. Both plasmid carrying strains reached the stationary phase around 2 h after induction of $sacB_{B.meg}$ expression with a final OD_{578nm} of 3. Levansucrase activity measurements were performed at all indicated time points. So far, the enzyme was not characterised with respect of polymerisation and hydrolysis activity. Hence, results of the activity test are given in grams of released sugars glucose and fructose per litre and hour (g l^{-1}

h^{-1}). The levansucrase genes *sacB*$_{B.meg}$ present on the vector pRBBm69 and in the genome had the identical sequence which was controlled by DNA sequencing. Hence, the gene products have the same amino acid sequence and catalyse the same reaction. Therefore, the amount of secreted protein and the release of sugars in the D.N.S. activity assay should be proportional. Within the first two hours, the amount of released sugars strongly increased from 0.4 to 3.9 g l^{-1} h^{-1}, when the *sacB*$_{B.meg}$ gene was present as a single copy gene on the chromosome, and from 5.0 to 44.3 g l^{-1} h^{-1}, when the *sacB*$_{B.meg}$ gene was present on a plasmid in addition to the chromosomal copy (**Fig. 35**). The same production profile was observed in *B. subtilis* secreting TEM β-lactamase under control of the sucrose inducible promoter P$_{sacB}$ of *B. subtilis* (Wong, 1989). The increased copy number of P$_{sacBB.meg}$-*sacB*$_{B.meg}$ present on the plasmid led to a 9-fold increase of SacB$_{B.meg}$ secretion at the beginning of the cultivation and slowed down to a 6-fold increase 6 h after induction of gene expression.

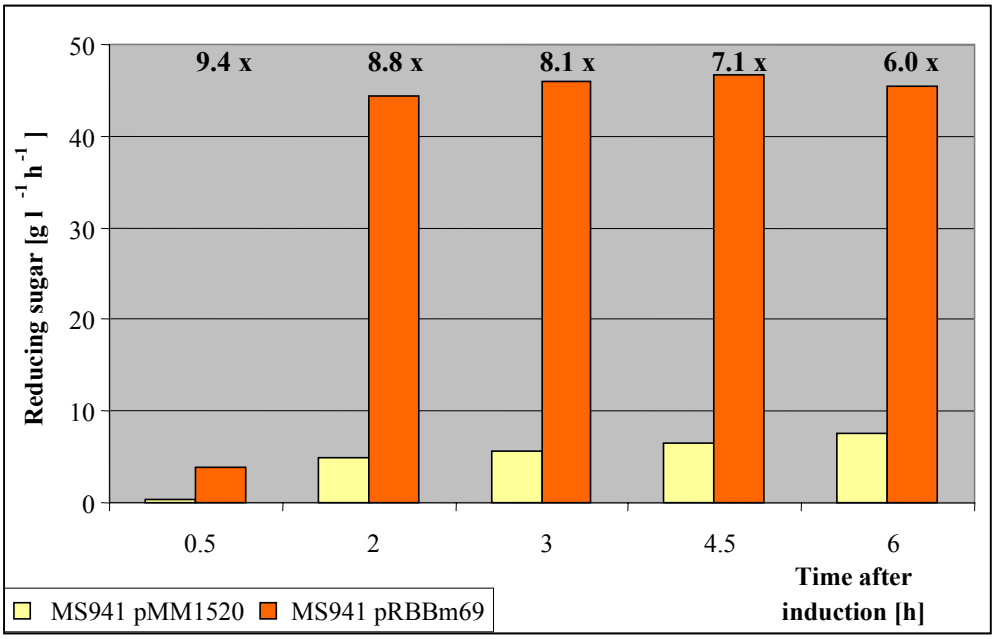

Figure 35: Levansucrase activity assay of medium of SacB*$_{B.meg}$* overproducing *B. megaterium*. *B. megaterium* MS941 transformed with pRBBm69 (encoding P$_{sacBB.meg}$-*sacB*$_{B.meg}$, orange bars) or target geneless pMM1520 (yellow bars) were cultivated aerobically in 100 ml LB medium at 37°C and 250 rpm. *sacB*$_{B.meg}$ expression was induced by the addition of 0.5 % (w/v) sucrose to the growth medium at an OD_{578nm} of 0.4. Samples were taken 0.5, 2, 3, 4.5, and 6 h after induction of the *sacB*$_{B.meg}$ gene expression. The levansucrase activity of the cell-free growth medium was determined using the D.N.S. method. Results are given in grams of released sugars glucose and fructose per litre and hour. Numbers above the bars represented the incremental factors. The background activity of the control strains MS941 transformed with pMM1520 results from chromosomally-derived *sacB* expression.

III.3.6 Induction Properties of the Sucrose Inducible Promoter $P_{sacBB.meg}$ of *Bacillus megaterium*

In 1991, the group of Wolfgang Hillen in Erlangen found the xylose isomerase gene *xylA* of *B. megaterium* under control of the xylose-inducible promoter P_{xylA} 200-fold induced in the presence of xylose (Rygus *et al.*, 1991). When they used the *B. megaterium* xylose-inducible promoter for overexpression of recombinant genes in *B. megaterium*, expression was induced up to 350-fold while adding xylose (Rygus and Hillen, 1991).

To characterise the sucrose inducible promoter $P_{sacBB.meg}$ with regard to the degree of induction, *B. megaterium* WH323 carrying target geneless pSTOP1622 and WH323 carrying pRBBm59 (encoding $P_{sacBB.meg}$-GFP) were grown aerobically in 100 ml of LB medium. The promoter $P_{sacBB.meg}$ was induced by the addition of 0.5 % (w/v) sucrose at an OD_{578nm} of 0.4. Negative control was WH323 carrying target geneless pSTOP1622 without addition of sucrose. The levansucrase activity of the cell-free growth medium was determined using the D.N.S. method 3 h after induction. Sucrose induction of $sacB_{B.meg}$ expression in WH323 carrying pSTOP1622 resulted in a 373-fold increase of reducing sugars glucose and fructose in the D.N.S. activity assay (9.69 g l^{-1} h^{-1}) and therefore of SacB production compared to the negative control (0.03 g l^{-1} h^{-1}). If sucrose was added to a cultivation of WH323 carrying pRBBm59 (encoding $P_{sacBB.meg}$-GFP), the additional GFP production led to a 55 % reduction of SacB secretion. But an 166-fold induction of $P_{sacBB.meg}$ (4.32 g $l^{-1}h^{-1}$) was still observed compared to the negative control (0.03 g l^{-1} h^{-1}) (**Fig. 36A**).

Finally, the induction of multi copy plasmid encoded promoter $P_{sacBB.meg}$ by sucrose addition was less intensive compared to the single chromosomal copy which was found to be 373-fold induced (**Fig. 36A**). Nevertheless, 54 times more $SacB_{B.meg}$ (46 g l^{-1} h^{-1}) was secreted if *B. megaterium* MS941 transformed with pRBBm69 (encoding $P_{sacBB.meg}$-$SacB_{B.meg}$) was cultivated aerobically in LB medium with sucrose addition compared to the negative control *B. megaterium* MS941 transformed with pRBBm69 (encoding $P_{sacBB.meg}$-$SacB_{B.meg}$) without sucrose addition (0.84 g l^{-1} h^{-1}) (**Fig. 36B**). An explanation could be the limiting number of regulatory proteins. Their target genes in the genome are present as single only while the promoter $P_{sacBB.meg}$ is encoded on a multi copy plasmid. Here, coexpression of the regulatory factors might further increase the amount of plasmid coded recombinant protein.

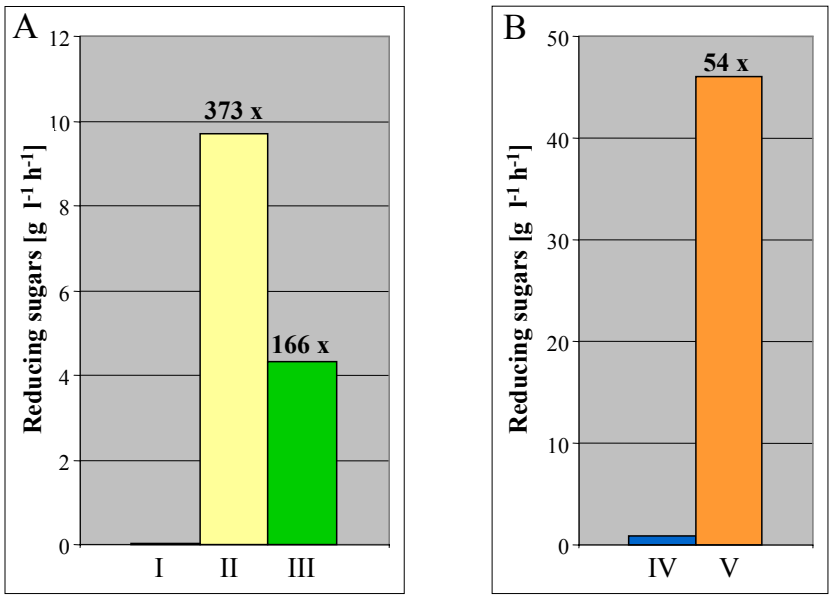

Figure 36: Induction properties of the sucrose inducible promoter in *B. megaterium*. (A) *B. megaterium* WH323 plasmid carrying strains were cultivated aerobically in 100 ml of LB medium at 37°C and 250 rpm. The $P_{sacBB.meg}$ promoter was induced by the addition of 0.5 % (w/v) sucrose at an OD_{578nm} of 0.4 in case of WH323 transformed with target geneless pSTOP1622 (II, yellow bar) and of WH323 carrying pRBBm59 (encoding $P_{sacBB.meg}$-GFP) (III, green bar). Negative control was WH323 transformed with pRBBm59 (encoding $P_{sacBB.meg}$-GFP) without addition of sucrose (I, black bar). **(B)** *B. megaterium* MS941 transformed with pRBBm69 (encoding $P_{sacBB.meg}$-$sacB_{B.meg}$) was cultivated aerobically in 100 ml of LB medium at 37°C and 250 rpm. $sacB_{B.meg}$ expression was induced by the addition of 0.5 % (w/v) sucrose to the growth medium at an OD_{578nm} of 0.4 (V, orange bar) while no sucrose was added in the cultivation of the negative control strain (IV, blue bar). **(A+B)** The levansucrase activity of the cell-free growth medium 3 h after addition of sucrose was determined by the D.N.S. method. Results are given in grams of released reducing sugars per litre and hour. Numbers above the bars represented the incremental factors.

IV SUMMARY AND OUTLOOK

IV.1 SUMMARY

In this thesis an alternative, competitive protein production and purification system was established using the Gram positive bacterium *B. megaterium*. For this purpose, a set of xylose-inducible gene expression vectors for the intracellular production of target proteins carrying combinations of N- and C-terminally fused His_6- and StrepII-affinity tags was constructed. These vectors were successfully tested using the green fluorescence protein (GFP) as a model. Up to 18 mg GFP l^{-1} were produced in shaking flask cultures and were purified to apparent homogeneity by affinity chromatography. High cell density cultivation allowed upscaling of the procedure to 274 mg GFP l^{-1}.

For the intracellular production and the subsequent export of target proteins containing various combinations of N- and C-terminally fused His_6- and StrepII-affinity tags into the growth medium, a second set of expression vectors was constructed. A *L. reuteri* levansucrase served as model for protein export studies. The efficiency of a *B. megaterium* lipase (LipA), penicillin G acylase (PGA), and a computer designed leader peptide for SEC-dependent protein export were evaluated. Up to 4.0 mg levansucrase l^{-1} were exported into the growth medium. The presence of affinity tags decreased the protein export. Coexpression of the gene *sipM* for the signal peptidase responsible for signal peptide removal increased the protein export. Deletion of the gene for the only detectable extracellular protease NprM stabilised exported proteins. Protocols for the purification of His_6- and StrepII-tagged exported target proteins from the growth medium were developed.

The alternative strong and sucrose-inducible $sacB_{B.meg}$ promoter system was identified using a secretome approach. This promoter and the $sacB_{B.meg}$ leader peptide were successfully applied for the high yield recombinant protein production and export. Finally, an expression system using two different, independently replicating plasmids was established and evaluated.

The established system provides an useful alternative to the commonly used *E. coli* systems. The majority of the developed vectors are now commercialised by the MoBiTec GmbH (Göttingen; Germany).

IV.2 OUTLOOK

In autumn 2004, the sequencing of the *B. megaterium* strain DSM319 genome has been started. The data available most likely in 2007 will open a new area for this production host. The complete annotation as well as the comparison of the data to other so far sequenced *Bacilli sp.* will be one of next very informative steps. This genome sequence provides the basis for a systems biotechnological analysis of *B. megaterium*. Transcriptome, proteome, secretome, and metabolome data will function as useful data for bioinformatics tools to construct first models of protein production relevant processes occurring in this organism. Deduced information may lead to the identification of general limitations existing in the *B. megaterium* system and will invite to further optimisation.

High throughput screening in industry as well as in research has become a wide spread tool for searching new products. The main limitation for the *B. megaterium* system is the low transformation rate. Now, the genome data will provide the possibility to find the missing components of the natural competence system present in *B. subtilis*. An easy and fast transformable bacterium with an excellent plasmid stability will lead to a broader application. Natural competence development will also facilitate the currently difficult gene knock-out in *B. megaterium*.

Intensified secretome analysis of *B. megaterium* cultures exposed to various nutrient sources (C-, N-, P-sources) or other extreme environmental conditions (osmotic stress, temperature, O_2) will help to identify further strong inducible promoters for a potential application in recombinant protein production. The established two vector system will provide the basis for a T7 RNA polymerase promoter system or the establishment of rare tRNA overexpressing (codon plus) strains.

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